THE ROLE OF EARLY NERVE-MUSCLE INTERACTIONS ON MUSCLE AND MOTONEURONE DEVELOPMENT.

A thesis submitted to the University of London for the degree of Doctor of Philosophy in the Faculty of Science.

by

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-July 1989-
To my parents in India and here in England.
ABSTRACT.

The importance of early postnatal nerve-muscle interaction for normal development is examined in this thesis. It has been found that temporary disconnection of muscles and motoneurones by nerve crush during early postnatal life permanently alters the properties of muscles and motoneurones.

The morphological and physiological changes that occur during early stages of reinnervation of neonatal fast and slow muscles are discussed in the first part of the thesis. Although the motor nerve reaches the fast extensor digitorum longus (EDL) muscle at the same time as the slow soleus muscle, the soleus muscle shows a more rapid recovery.

The permanent effects on the fast muscles tibialis anterior (TA) and extensor digitorum longus (EDL) of interrupting interaction with their nerves during early postnatal life by nerve crush 3 or 9 mm away from them are discussed. The results show that the shorter the period of interruption of nerve-muscle interaction during early postnatal life, the better is the recovery of reinnervated muscles.

The effects of neonatal sciatic crush on the efferent inputs to surviving motoneurones after reinnervation has been studied. Stimulation of efferent nerves ipsilaterally or contralaterally produces a greater reflex response in the reinnervated than in the control muscle, indicating that the activation of motoneurones is changed by neonatal nerve injury.

In the final part of the thesis the possibility of the involvement of calcium in the death of 50% of motoneurones of the sciatic pool after sciatic nerve crush at birth has been studied. The results show that a calcium chelating agent at the site of the nerve injury marginally improves motoneurone survival. The possibility that this may be due to local effects on regenerating axons is discussed.
ACKNOWLEDGEMENTS.

To my supervisor and mentor, Professor Gerta Vrbová, my very special thanks. Without her this work would not have been possible. She has been a source of inspiration and imagination which is hard to match. Also unique is her warmth and vitality which permeates within the group making work a pleasure.

Thanks go to my colleagues Angela Connold, Linda Greensmith, Angela Hind and Rolf Frischknecht who provided a mutual support system in times of stress. I am very grateful to Dr. Maggie Lowrie for her patience and forbearance in answering my queries. I should like to acknowledge Dr. Roberto Navarrete and Diana Phillips with whom some of the work presented in this thesis was done. It was a pleasure working with them.

Many thanks also to Denny John for lending me his Apple MacIntosh to draw the figures presented here.

Finally, my heartfelt thanks go to all my friends particularly to David, Theo and Colin Mansfield and to Kay Bicket for their unstinting moral support and love throughout this venture.
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CHAPTER 1.

General Introduction.

In mammals coordinated movement is based on an orderly recruitment and function of motor units. A motor unit consists of the motoneurone and the muscle fibres it innervates. Since motoneurones and muscle fibres act as units, it is important for their characteristic properties to match. The development of this matching is an important part of the maturation of the neuromuscular system and is achieved by interactive processes between the motor nerves and muscles. The main purpose of the experiments discussed in this thesis has been to study the role of the interaction between muscles and their motoneurones during postnatal development.

An overview of the literature relating to embryonic and postnatal development of muscles and motoneurones and their mutual interdependence will be considered in Part I.

The effects of temporary denervation on adult and neonatal neuromuscular systems will be discussed in Part II.

Part I.

1.1.1. Early development of nerve and muscle.

In the spinal cord of vertebrate embryos, motoneurones are the first neurones to emerge and migrate to their appropriate targets (Cajal, 1928). Muscle cells develop from mesenchymal cells that are committed to becoming myoblasts. These cells fuse with each other to form myotubes. It is at this stage that the first exploring axons reach their target muscles and begin to make synaptic contact with the myotubes. Until this time motoneurones and muscles develop independently from each other. It is only now, as the first
neuromuscular contacts are made that they acquire an interdependence which enables them to interact as a functional unit (for review see Vrbova, Gordon and Jones, 1978).

a) Early development of the Motoneurone.

The first neurones to emerge and migrate to their appropriate positions are the motoneurones (Cajal, 1928). They are formed from neuroblasts and migrate from the central canal to the ventral horn (Cajal, 1928; Fujita, 1963; Jacobson, 1970). At this stage removal of the peripheral target does not stop the outgrowth of the axons that form the ventral roots (Hamburger, 1958; Hughes and Tschumi, 1958; Oppenheim, 1978). In the rat, ventral roots are first seen at day 11 or 12 of gestation and in the mouse, axons from the ventral roots extend to the hindlimb-buds at 15 days (Filogamo and Gabella, 1967; Platzer, 1978; Lance-Jones, 1982).

b) Early development of muscle and its dependence on the motoneurone.

During the period when the nerves reach their targets, muscle cells have already begun their development from mesenchymal cells that are committed to being myoblasts. In the mouse this is known to happen at embryonic day 13 (Platzer, 1978). In several species, fusion and intracellular differentiation of myoblasts to form myotubes in several species coincides with the arrival of these exploring axons. Muscle specific proteins can be synthesised by myoblasts (Holtzer et al., 1957; Shainberg et al., 1971; Fambrough and Rash, 1971; Holtzer et al., 1973; Dryden et al., 1974). Once myoblasts stop dividing and fuse into myotubes and start contracting, a rapid increase in these muscle specific proteins is seen (Holtzer, 1967; Fambrough and Rash, 1971; Giacobini et al., 1973; Whalen et al., 1976). At this time, application of acetylcholine (ACh) to the developing muscle cell,
either diffusely or by intophoresis, causes depolarization of the membrane and the contraction of myotubes (Dryden, 1970; Fambrough and Rash, 1973; Dryden et al., 1974), which suggests the presence of Ach receptors to be among the first manifestations of muscle differentiation. Myotubes continue to differentiate and increase in size and complexity. Large and small myotubes referred to as primary and secondary myotubes respectively form clusters and are enclosed within the same basal lamina. As development proceeds, the secondary myotubes acquire their own basal laminae and become independent fibres (Kelly and Zacks, 1969a; Ontell, 1977).

The tropomyosin-troponin complex and the sarcoplasmic reticulum (SR) are seen to develop next (Hitchcock, 1970; Luff and Attwood, 1971). Myofibrils and sparse SR are seen by day 14 or 15 of gestation in mouse hindlimb muscles (Platzer, 1978). By embryonic day 16, myofibrils align themselves and form sarcomeres. This coincides with the first spontaneous movements of the mouse embryo (Kelly and Zacks, 1969a; Platzer, 1978). Myotubes continue to differentiate after birth, increasing in size and complexity (Ontell and Dunn, 1978; Dennis et al., 1981; ), but for the further development of myotubes contact with the axons is essential. If the myotubes are not innervated beyond the stage of primary myotube formation, their differentiation is arrested and they atrophy (Hamburger, 1939; Eastlick and Wortham, 1947; Drachman, 1968; Harris, 1981). The stunted development seen in non-innervated muscles can also be induced by neuromuscular blocking agents such as curare, botulinum toxin, hemicholinium and suxamethonium (Drachman, 1964; 1968; Gordon et al., 1974; Giacobini-Robecchi et al., 1975; ). Thus, at a certain stage of development
neuromuscular interactions seem essential for the normal maturation of muscle cells as well as their innervating motoneurones.

1.1.2. Retrograde influence of the target on the development of motoneurones.

Evidence from the chick embryo shows that the initial stages of neurone differentiation are unaffected by the removal the target limb bud (Hamburger, 1958). Beyond this stage of development when nerve fibres grow out to the periphery, unless contact is made with the targets, motoneurones in the ventral horn disperse and degenerate. However, during normal development several motoneurones degenerate and die despite the presence of the target, once they establish contact with it (Hamburger, 1975). The first conclusive study that showed that large scale cell death was a naturally occurring phenomenon during the embryonic development of the vertebrate nervous system came from chick embryos, where degeneration of dorsal root ganglia was observed (Hamburger and Levi-Montalcini, 1949). Levi-Montalcini (1950) also noted similar death among motoneurones in the cervical part of the chick spinal cord. It is now known that this phenomenon affects all types of neurons (Oppenheim, 1981; Cowan, 1984; Oppenheim, 1985; Purves and Lichtman, 1985).

Motoneurone death occurs after all the cells have migrated. In chicks, 40% of the cells die (Hamburger, 1958; 1975). In amphibians the percentage loss of neurones is even greater, between 80 and 90% and coincides with the first spontaneous movements of the embryo (Prestige, 1967; Hughes, 1968). In mammals, for example mice, 40 to 75% of motoneurones die between day 12 and 15 of gestation (Harris-Flanagan, 1969; Lance-Jones, 1982).
Two possibilities as to the function of the generalised phenomenon of cell death seen in vertebrate embryos have been proposed.

a) Cell death was thought to occur in order to remove errors of connectivity (Hughes, 1968).

b) The fact that the system could afford such errors seemed to suggest that the developing nervous system provides redundancy to ensure complete innervation of the target (Hamburger, 1977).

Both these possibilities are interrelated. The experiments that showed a larger decrease in motoneurone number after limb bud removal or an increase in their number after grafting of a supernumary limb onto an existing limb bud (Hamburger and Levi-Montalcini, 1949), suggested to the experimenters that motoneurones were in competition with each other for synaptic sites on the target. Hence it was thought that regulation of cell death and removal of redundancy may be dependent on competition for some "trophic" substance released by the targets that would allow the maintenance of synaptic contacts (Hamburger and Levi-Montalcini, 1949; Hamburger, 1975).

This idea cannot however explain other experimental findings. Lamb (1980) found that motoneurones survived even when an excess number of neurones were directed towards relatively little muscle tissue. If a shortage of growth factors were responsible for motoneurones dying, then fewer motoneurones should have survived the experiment described above and this was not the case. Harris, (1981), and McLennan, (1982) found that there was an increased number of myotubes and myofibres during motoneurone death suggesting that there was no lack of synaptic sites for the dying motoneurones to go to. However, McLennan, (1982) still proposed the idea that it was this very quantitative mismatch
that precipitated cell-death. But once again, this could not be due to limited synaptic sites because it is known that myotubes and myofibres in normal and experimental situations (Bennet, 1983) accommodate inputs from more than one motoneurone. Unpublished observations of Oppenheim and Chu-Wang (see Oppenheim, 1987) have shown that motoneurone death coincided with the net increase in neuromuscular synapses. So, despite the increased number of synapses, no reduction in cell-death was seen.

Several experimenters thought that the electrical activity of other neurones, afferents, and targets may regulate the process of cell-death (Pittmann and Oppenheim, 1978;1979; Olek, 1980; Ding et al., 1983; Harris and McCaig, 1984; Fawcett et al., 1985) in conjunction with the release of some "trophic" factor. Based on the conceptual arguments of Changeux and Danchin (1976) that cell-death occurred due to the formation of non-functional synaptic contacts,(see Oppenheim, 1987) they devised an experiment in which they reduced neuromuscular activity by pharmacological agents and neurotoxins such as curare and alpha-bungarotoxin during the period of normal motoneurone death in chick embryos. Contrary to their expectations, cell death was temporarily prevented and not increased by paralysis. The converse was true when the limb bud was electrically stimulated. (Pittmann and Oppenheim, 1979; Oppenheim, 1984). Thus, neuromuscular activity seemed to be directly related to motoneurone death.

However, it has been argued that in these experiments motoneurones were rescued by a variety of pharmacological agents which block synaptic transmission centrally within the spinal cord (Landmesser, 1984). But curare treatment did not rescue motoneurones
after limb bud removal (Oppenheim et al., 1978). Furthermore, inhibitory neurotransmitters such as γ-aminobutyric acid (GABA) which act centrally (and not at the neuromuscular junction) by reducing the movements of the chick embryo, (Reitszel, et al., 1979) if administered chronically, during the period of motoneurone death, do not succeed in preventing the phenomenon (Oppenheim, unpublished observations). Although neuromuscular activity appears to be crucial in inducing cell-death, some evidence suggests that a few motoneurones can be rescued if they are treated with dibutyryl cyclic GMP and sera (IgG) from patients with myasthenia gravis without affecting normal neuromuscular activity (Sohal et al., 1983; Weill and Greene, 1984). It would appear from these results that suppression of neuromuscular activity may be a sufficient but not prerequisite factor in rescuing motoneurones from death. Certainly, some authors have argued that it may only be one link in a complex chain of events that regulate neurone survival or death (Hamburger and Oppenheim, 1982; Oppenheim, 1987). In this context, these authors have argued that neuromuscular activity releases a putative "trophic" factor so that only a few motoneurones have access to it. The release of this "trophic" factor they argue is related to the localisation of acetylcholine receptors (ACh-Rs) at the neuromuscular junction that occurs as the muscle becomes innervated. Paralysis by curare for example, may spread the ACh-Rs all over the surface of the muscle thus "de-localising" them as is the case when a muscle is denervated (see next section). This would enhance the chances of those motoneurones that would normally have had to compete for the putative trophic factor to gain access at the neuromuscular junction (see Oppenheim, 1987;1989). Whatever the mechanism of cell-death, most of the evidence so
far implies that interaction between the motoneurone and its muscle is crucial to normal development. To understand this process of interaction better, it is necessary to review the development of the neuromuscular junction and this will be considered in the next section.

1.2.1. Development of the neuromuscular junction

When axons of young motoneurones reach the limb bud it is predominantly composed of myoblasts. These cells already have some muscle specific proteins, they synthesize acetylcholine receptors (AChR) but are still unresponsive to nerve stimulation (Fishman, 1972; Fambrough, 1979). Studies on cultured chick myoblasts show that AChRs are unevenly distributed along the muscle membrane forming "hot spots" (Fambrough and Rash, 1971; Fischbach and Cohen, 1973). When myoblasts form myotubes they become innervated (Tello, 1917). At the onset of neuromuscular contact the myotube membrane undergoes certain changes at the place of contact. These include the localisation of the AChR and the accumulation of Ach at the site where the axon contacts the muscle (Couteaux, 1963; Hirano, 1967; Fambrough, 1979) and immature endplates are formed. These have few specialisations. Their nerve terminals have few synaptic vesicles and mitochondria and their postsynaptic membranes do not have the folds seen at adult endplates (Couteaux, 1963; Kelly and Zacks, 1969; Juntunen, 1979). Despite the lack of synaptic differentiation the newly formed neuromuscular junction can transmit the impulse from the nerve ending on to the muscle (Strauss and Weddell, 1940).

Evidence of the first synaptic activity is observed between days 14 and 16 of gestation in the rat (Diamond and Miledi, 1962; Bennet
and Pettigrew, 1974; Dennis et al., 1981). Miniature end-plate potentials (MEPPs) are common at this stage and their low frequency suggests that the rate of spontaneous transmitter release is small (Diamond and Miledi, 1962). End-plate potentials (EPPs) and action potentials (APs) can also be recorded from immature muscle fibres (Kullberg, 1977) but prolonged repetitive stimulation results in failure of neuromuscular transmission (Letinsky, 1974; Pilar et al., 1981). This immaturity of synaptic transmission reflects the low levels of transmitter synthesis and cholinergic enzymes by the nerve (Chiappinelli et al., 1976; O’Brien and Vrbova, 1978; Pilar et al., 1981). But the high input resistance and small diameter of myotubes along with the low rates of degradation of ACh by acetylcholine esterase (AChE) allow neuromuscular transmission during early development (Dennis et al., 1981).

Functional activity precedes the development of specific morphological features of the neuromuscular junction. This suggests that neuromuscular transmission itself plays an important role in the maturation process. Experimental evidence testifies to this.

Differentiation of the postsynaptic membrane and development of the subneural apparatus is arrested when neuromuscular transmission is chronically blocked in the chick embryo during the period of synapse formation (Gordon and Vrbova, 1974; Srihari and Vrbova, 1978). Motor paralysis produced by Flaxedil for example results in a specific failure of the heavy or junctional form of AChE to appear (Betz et al., 1980).

Neuromuscular activity is also responsible for the changes of ACh sensitivity along the muscle membrane. Embryonic and neonatal muscle fibres are sensitive to ACh along their entire surface. This becomes
restricted to the endplate region as development proceeds and fails to occur if innervation is absent. Inactivity prevents the restriction of AChRs to endplate regions (Gordon and Vrbova, 1975) and indeed in the chick embryo treated with curare or alpha-bungarotoxin, an increase of AChR clusters is seen on myofibres (Oppenheim and Bursztajn, 1983). This is consistent with the idea that continued neuromuscular activity is vitally important during development.

At the neuromuscular junction, the AChR is known to change as a result of development. This has been studied in terms of the half-life of the receptor and the mean opening times of associated ion channels. Initially, prior to innervation, all the AChRs have a rapid turnover rate and the half-life of each is 18 to 22 hours. Once cluster formation has occurred at between 17 and 19 days of gestation in rat skeletal muscle, the junctional turnover rate falls to give a half-life of 9 to 11 days (Reiness and Weinberg, 1981).

Changeux and Danchin (1976) attributed this change to a "nerve-factor" which they said could be released from the terminal to induce a state of stability in the AChR. No release of any such factor has been reported, but recent evidence has suggested that muscle activity in response to nerve stimulation induces changes in the accumulation and stabilization of the AChRs such that resultant changes occur in their number and junctional properties (Lomo and Slater, 1980; Lomo, Mirsky and Pockett, 1984; see Salpeter and Loring, 1985 for review).

Receptor-associated ion channel properties also develop and alter as maturation proceeds. Developing synapses have channels with a mean open time of 4.6 milliseconds. This decreases to 1 millisecond at adult end-plates. This change occurs 8 to 18 days postnatally in the
rat soleus muscle (Sakmann and Brenner, 1978; Fischbach and Scheutze, 1980; Brenner et al., 1987).

Evidence indicates that neuromuscular activity is involved in inducing the decrease seen in the channel opening times at the endplate. Denervation of the soleus shortly after birth results in the delay of transformation of "fast" channels (Scheutze and Vicini, 1984). In contrast, the fast gating properties of AChR channels of denervated muscles of adult rats are maintained for upto 3 weeks or longer after denervation in the end-plate membrane (Brenner and Sakmann, 1983). Results reviewed here suggest that neuromuscular activity is essential to change the AChR properties from immature to adult patterns during muscle development.

1.2.2. Synaptic transmission in developing muscles.

MEPPs which occur because of the spontaneous release of ACh from the nerve have been observed at day 14 of gestation in rat intercostal muscles (Dennis et al., 1981). Their frequency is very low before birth (10^-3 to 10^-4 per second) but increases postnatally to about 1 to 2 per second as adult patterns of locomotor activity are established (Diamond and Miledi, 1962; Dennis et al., 1981). These foetal MEPPs have several characteristics similar to their adult counterparts. For example, they increase in frequency when exposed to raised extracellular potassium or increased osmolarity (Dennis et al., 1981). In contrast to adult MEPPs they have a slow rise time and rate of decay which only increases as AChR and AChE accumulate at the end-plate. The different time course seen in the MEPPs of immature muscles may be due to different membrane constants in the myotubes and to their electrical coupling (Diamond and Miledi, 1962; Bevan and Steinbach, 1977; Braithwaite and Harris, 1979).
EPPs occur as a result of nerve stimulation and have been recorded at the same time as MEPPs are seen in the rat embryo (day 14 of gestation) from the intercostal muscles. They can be large enough to cause muscle contraction but they are similar to MEPPs in their slow rate of rise and decay and are also affected by electrical coupling between the myotubes.

Kelly and Zacks (1969a and b) reported that only a small area of contact is seen between the muscle and nerve with few synaptic vesicles in the presynaptic nerve terminal. Repeated nerve stimulation at low frequencies causes a decrease in the amount of transmitter released and this is seen in the reduced size of individual EPPs. Lowrie et al., (1985) also found that the quantal content at rat soleus end-plates increased postnatally as the animal matured. Thus, it appears that immature nerve endings have a small amount of transmitter available for release.

Another interesting feature of developing muscle is the initial formation of excess nerve contacts at each endplate so that at and shortly after birth, each skeletal muscle fibre is contacted by axons from several motoneurones. This situation outlasts motoneurone death and carries on into postnatal life until it is gradually modified to the adult situation in which one axon contacts one muscle fibre. Motoneurones, however, continue to supply many muscle fibres whereas each muscle fibre has only one input from an axon of one motoneurone. The removal of excess terminals from young muscle fibres to achieve the adult status is a result of maturation and seems to be dependent on the activity and interaction between motoneurones and muscles.
1.2.3. Polyneuronal innervation: Its existence and elimination.

It was Redfern in 1970 who first described the existence of polyneuronal innervation in the rat diaphragm. Since then, several other studies have shown that polyneuronal innervation in rats and other mammals can be detected by both histological techniques of visualising axons and endplates or electrophysiological ones by recording compound EPPs (Redfern, 1970; Riley, 1977b; O'Brien et al., 1978; Connold et al., 1986; Evers, 1987; Connold, 1989). The compound EPPs seen electrophysiologically at a single endplate cannot be attributed to the electrical coupling of myofibres seen during early development, since this is known to have disappeared shortly after birth (Kelly and Zacks, 1969a; Dennis et al., 1981).

Evidence that the axons innervating single muscle fibres come from individual motoneurones and meet at the same endplate was first presented by Bagust and his colleagues (1973) with the experiments on stimulation of ventral root filaments. Later evidence has confirmed this finding that axons in young muscle fibres are indeed from different motoneurones by showing the proportion of overlap of ventral roots innervating the soleus muscle. Thus, the maximum tetanic tension produced by both L4 and L5 ventral roots is less than the sum of the tension produced by stimulating each root individually (Brown et al., 1976; Miyata and Yoshioko, 1980). Riley (1976), found that only a small proportion of axons seen innervating a single endplate in young muscles were branches of individual axons belonging to the same parent motoneurone.

The mechanism of elimination of supernumary terminals to achieve the adult state of a one to one relationship between the motoneurone
and its muscle fibre involves a numerical reduction in the ratio of terminals to muscle fibres and there may be several ways in which this could be brought about. The most simple way of achieving this goal would be either by increasing the number of muscle fibres for the excess terminals to innervate or by the death of the parent neurone, in which case the axon and its terminal would retract and die too. However, removal of excess motoneurones which is a normal part of development, is already complete at least in avian embryos (Holt and Sohal, 1978) and precedes elimination of excess nerve terminals by several days. In some mammals however, naturally occurring motoneurone death may overlap with the early stages of elimination. For example, Nurcombe et al., (1981) reported that 45% of neurones in the brachial motor column of the rat died between days 1 and 4 after birth but there was very little loss after this time although elimination continued until 10 or 12 days later. The authors suggested that motoneurone death could account for the loss of some of the terminals or that the loss of terminals caused motoneurone death. However, the results of these experiments were proved wrong with subsequent evidence. In addition, at least in the soleus muscle of the rat, Brown et al., (1976) found that there was no reduction in the number of axons innervating the muscle, and that elimination of terminals could not be responsible for motoneurone death.

The loss of terminals and the elimination of polyneuronal innervation could be explained by a redistribution of the excess terminals onto new muscle fibres that may be formed after birth. However, in the rat at least, most of the muscles of the lower limb have been found to have their full complement of muscle fibres at birth (Ontell and Dunn, 1978; Betz et al., 1979; Ontell, 1979). Muscle
fibre numbers increase after birth in the fourth lumbrical muscle of the rat but, despite this increase, and the redistribution of excess terminals that occurs onto these new fibres up to 10 days postnatally, it has been found that loss of terminals continues to occur up to 6 days after the cessation of production of new muscle fibres (Betz et al., 1979). The mechanism accounting for the transfer of terminals from one fibre to another still needs to be explained however, and the process by which the excess terminals are removed from each end-plate may account for the net loss of terminals seen.

Evidence both histological and electrophysiological seems to suggest that the extra axons retract into the parent axons (Redfern, 1970; Brown et al., 1976; Korneliussen and Jansen, 1976; Ostberg and Vrbová, 1977; Riley, 1977b; Bixby, 1981; Haiman et al. 1981b; Riley, 1981). The mechanism that accounts for the retraction of the terminals remains elusive although several models have been proposed. However, before these are discussed, it would be prudent to assess the role of the increased locomotor activity of young animals.

It is now generally accepted that the elimination of excess terminals coincides with increased locomotor activity of the developing animal (Navarrete and Vrbová, 1983) and is delayed by a reduction in nerve activity by tenotomy, spinal cord transection or by paralysing agents such as tetrodotoxin (Benoit and Changeux, 1975; Thompson et al., 1979, Caldwell and Ridge, 1983). The process of elimination can also be hastened by inducing an increase in neuromuscular activity (O’Brien et al., 1978, Zelená et al., 1979). That an active target is crucial to the process of elimination of excess terminals and retraction of axons is clear from experiments in
which postsynaptic neuromuscular blocking agents such as curare and alpha-bungarotoxin which cause paralysis, retard or even prevent the process (Srihari and Vrbová, 1978; Sohal and Holt, 1980; Duxson, 1982). Thus, it is clear that the activity of the target is essential if synaptic re-organization has to take place. The mechanism by which this takes place is still not clearly understood but evidence from our laboratory suggests that a calcium-activated neutral protease (CANP), known to be present in nerves (Kamakura, et al., 1983), plays an important role in the elimination of superfluous terminals in the developing animal (O’Brien et al., 1978; 1980; 1984; Connold et al., 1986; Evers, 1987; Vrbová et al., 1988; Connold, 1989). A model has therefore been proposed as to how this might be. Briefly, it is as follows: The process is dependant on the activity of the postsynaptic membrane which releases potassium ions to open voltage-dependent calcium channels of nerve terminals. These channels are slowly inactivating and would remain open for long periods of time. Consequently, the entry of calcium in small terminals could be high enough to activate the CANP which would destroy the cytoskeletal proteins of those nerve terminals causing them to withdraw and disruption of neuromuscular contact will occur. That the potassium concentration is high in the synaptic cleft as a result of neuromuscular activity has already been shown (Hohlfield et al., 1981). Additionally, increasing extracellular potassium to 20 mM for just 2 hours in the medium in which rat soleus muscles were incubated reduced polyneuronal innervation by 20% as compared with its contralateral control not incubated in high potassium medium. Treating muscles with CANP inhibitors had the opposite effect (see Connold et al., 1986; Vrbová et al., 1988; Connold, 1989). Considering this
evidence, it has been proposed that smaller terminals would be more vulnerable to calcium entry because, in view of their surface to volume ratio the relative calcium concentration should increase more in them than in the larger terminals. Thus, the more active the young muscle, the more likely it would be to eliminate excess contacts.

1.3.1. Development of Spontaneous and reflex activity.

This section will review some of the literature concerned with the development of spontaneous and reflex movements during early life. It is known that mammalian embryos are at a more advanced stage of development than chick embryos when the first spontaneous movements are seen. Using light microscopical and ultrastructural findings Windle and Baxter (1936), and Vaughn and Grieshaber (1973) have shown that segmental and intersegmental reflex circuits in rat and cat embryos have already been established and are functional at the time the first movements are known to appear.

It has been suggested that the formation of connections between neurones occurs in retrograde sequence (Cajal, 1928; Vaughn and Grieshaber, 1973). Thus, motoneurones develop prior to any other neurones in the rat spinal cord. Interneurones are the next cells to develop. Monosynaptic connections from spindle afferents to motoneurones develop last of all on the 18th or 19th days of gestation (Grobstein, 1979).

Behavioural observations of the first neurogenic movements in the rat foetus have been observed at 15 or 16 days of gestation and consist of lateral flexion of the head (Angulo y Gonzalez, 1932; Windle and Baxter, 1936). The development of movement follows a cranio-caudal sequence as in other mammalian and non-mammalian species.
(Barron, 1941) and applies to forelimb and hindlimb movement with a few exceptions (Narayanan, et al., 1971).

Three basic kinds of movements have been observed in rat embryos at different stages of development. They are as follows: a) There are generalised mass movements which involve the whole foetus and which predominate between days 16 and 17 of gestation. They are irregular spontaneous movements of the head, neck and forelimbs. Activity is unevenly distributed in the foetus during such movements. b) There are partial or regional movements which are still generalised but only over a given part of the foetus at any one time. These occur later once the generalised mass movements have already begun. c) These generalised regional movements are followed by specific localised movements such as flexion of a single limb (Narayanan, et al., 1971). As development proceeds, these predominate and mass movements become less frequent.

Quantification of spontaneous activity in rat embryos shows that it increases from 20% at 16 days of gestation to a peak of 48% at 18 days. It then decreases to 35% by the end of the period of gestation, at 20 days in utero. This sequence has also been observed in chick embryos but over a longer period of time. In general, after birth, spontaneous activity decreases dramatically and this may be due to the activation of supraspinal inhibitory inputs modulating it (Barron, 1941).

Some features of spontaneous activity which are characteristic to embryonic life, continue to be expressed in early postnatal life in mammals. For example, bursts of phasic activity are seen in newborn rats usually in paradoxical sleep. These are thought to be a
continuation of autogenic embryonic movements (Corner, 1977). However, the change from the protected environment in utero to the challenging one outside of it places different demands on the young motor system.

Thus, it is not surprising that observations of locomotor activity in young rats for example, show that the motor system is still immature (Stelzner, 1971; Altman and Sudarshan, 1975; Oakley and Plotkin, 1975). The main kind of spontaneous activity observed at birth consists of use of forelimbs. This progresses to crawling during the second week and walking by the third week (Altman and Sudarshan, 1975). Adult patterns of muscle activity are seen to be established by the third week (Navarrete and Vrbová, 1983) and coincide with several other features of development such as opening of the eyes. Maturation of synaptic connections and reflex activity in the spinal cord also coincides with the increase in locomotion seen by the third week of life (Stelzner, 1971; Gilbert and Stelzner, 1979). Behavioural observations of motor reflexes in foetuses and neonates has shown that stimulation of specific nerves results in the generalised non-specific response of the whole animal. This becomes more restricted to that part of the body that is stimulated as development proceeds and adult patterns of activity are reached (Windle and Baxter, 1936; Windle, 1940; Barron, 1941).

Electrophysiological evidence from new born kittens has shown that enhanced responsiveness to cutaneous stimulation may be due to the absence of inhibitory mechanisms in the spinal cord that only develop later (Malcolm, 1953). Skoglund, (1960a) and Ekholm (1967) have shown that cutaneous stimulation produced simultaneous excitatory effects in both flexor and extensor hindlimb muscles. During postnatal develop-
ment this becomes progressively restricted and alters to the characteristic reciprocal effects on antagonist muscles that is seen in adult animals. Additional confirmation of this comes from experiments by Navarrete and Vrbova (1983), in which they have described the development of different firing patterns of slow and fast muscles. These have suggested that the functional properties of motoneurones are still differentiating until about the third week of life.

Postural reflexes such as the tonic stretch reflex seem to develop later than cutaneous reflexes at least in the distal hindlimbs of mammals (Skoglund, 1960a). Although monosynaptic connections between proprioceptors and motoneurones are present at birth (Skoglund, 1960b), tonic discharges from muscle spindles cannot be recorded (Skoglund, 1960c). The maturation of the stretch reflex takes place as the conduction velocity of spindle afferents increases with the development of tonic discharges from these fibres (Skoglund, 1960d).

In the rat foetus, spinal reflexes have been shown to be elicited initially as early as 16 to 18 days of gestation, from a wide range of segmental and intersegmental afferents (Saito, 1979), and these reflexes become more restricted during early postnatal life (see Fitzgerald, 1985). Recent results using a spinal cord-hindlimb preparation in vitro have shown that in neonatal rats (upto 3 days old), stimulation of either flexor or extensor muscle nerves produced a short latency co-excitatory reflex EMG (electromyographic) response in both the agonist and antagonist muscles (Navarrete, Walton and Llina, 1987). The occurrence of this co-activation was age-dependent and decreased to about 30% of the motoneurones sampled between days 8 and 10 after birth (Navarrete and Walton, 1988). This is very different to the reciprocal inhibition seen in adult animals. It is
obvious from the evidence discussed above that motor reflexes are still immature in young animals and develop as functional connections between neurones are gradually established. Patterns of muscle activity reflect the state of development of afferent systems, motoneurones and the interneurones mediating their responses. The next section will review the evidence relating to motoneurone properties and their connections within the mammalian spinal cord.

1.3.2. Motoneurone properties and connections.

Neonatal motoneurones, for example in the rat spinal cord, have been shown to be immature and their synaptic connectivity and intrinsic electrophysiological properties are different from those of adult motoneurones. They have high input resistance and low maximum firing rate compared to adult motoneurones (Fulton and Walton, 1986). They are smaller and also more easily excited by afferent stimulation (Mellstrom and Skoglund, 1969; Kellerth et al., 1971).

Kellerth et al., (1971) showed that all neonatal motoneurones of kittens have very similar electrophysiological properties and none of the differences between the firing patterns of "slow" and "fast" motoneurones that they mature into exists. The differences in the motoneurones were only thought to develop as adult patterns of activity were expressed. At this point a brief description of the properties of adult motoneurones is necessary in order to compare them with those of neonatal motoneurones. The first description of the firing patterns of adult motoneurones came from the classical experiments of Denny-Brown (1929a,b). He showed that motoneurones to slow muscles (for example, soleus), fired at low rates for prolonged periods in response to the stretch reflex, whereas motoneurones to
fast muscles (for example tibialis anterior) fired briefly at higher rates. The former had low thresholds of recruitment and the latter had high thresholds of recruitment. Several later studies confirmed these observations (see Burke, 1981).

Huizar et al., (1975) confirmed the findings of Kellerth et al., (1971), by studying the electrophysiological properties of motoneurones supplying "slow" soleus and "fast" gastrocnemius muscles in kittens. They found that to begin with the afterhyperpolarization of the action potential was short in both the types of motoneurones mentioned. As development proceeded, only the motoneurones to the soleus muscles showed an increase in their afterhyperpolarization. Conduction velocities of both types of motoneurones increased with age however (Ridge, 1967, Huizar et al., 1975).

Recent studies on rat neonatal motoneurones are in agreement with those on kitten motoneurones by Huizar et al., (1975). Three-day-old rat motoneurones were found to be clearly distinguishable from those of slightly older animals aged between 8 and 10 days (Navarrete and Walton, 1988) by their marked afterdepolarization and afterhyperpolarization. These latter characteristics reflect the presence of prominent calcium and calcium activated potassium conductances respectively (Walton and Fulton, 1986). However, in both presumptive fast and slow motoneurones a decrease in the afterhyperpolarization and input resistance has been seen with age. This latter, is indicative of the differentiation of the cells with respect to these two parameters that continues beyond 10 days of age.

Postnatal growth of the motoneurone's soma has been found to be proportionately less than that of axon diameter. Thus, proximal parts of the motoneurone may attain maturity earlier than distal parts
The significance of these changes is not clear in normal development and discharge of motoneurones because their activity depends not just on size but also on the source and distribution of synaptic inputs.

Morphological and physiological evidence exists to show that some synaptic connections develop in the first few weeks of postnatal life. For example, the cortico-spinal tract has been shown to be functional 9 to 14 days after birth (Hicks and D'Amato, 1975). In the lumbar spinal cord synaptogenesis is maximal around the time of birth and the development of descending and intrinsic spinal connections is complete only by the third week of postnatal life (Stelzner, 1982). Although interneuronal synapses onto motoneurones are formed early in embryonic life and primary afferent monosynaptic connections to motoneurones occur just before birth (Kudo and Yamada, 1987), evidence exists that upto 50% of synapses onto motoneurones are eliminated during the first 3 weeks of postnatal life in kittens (Conradi and Ronnevi, 1975). Thus, it is possible that during early postnatal development new synapses onto motoneurones are being formed while others are being eliminated. Some evidence indicates that these synaptic rearrangements may be due to elimination of axon collaterals (Culheim and Ulfhake, 1982). But more recent evidence on spinal reflexes suggests that there may be synaptic rearrangements within the spinal cord itself. Initially, spinal reflexes can be elicited from a wide range of segmental and intersegmental afferents (Saito, 1979) and these reflex inputs become restricted during postnatal development (see Fitzgerald, 1985). Recent results using an in vitro spinal cord-hindlimb preparation have shown that in neonatal rats (upto 3 days old)
stimulation of either flexor or extensor muscle nerves evoked a short latency co-excitatory reflex EMG response in both agonist and antagonist muscles (Navarrete, Walton and Llinas, 1987). This contrasts with the reciprocal inhibition seen in adult animals.

Thus, it is possible that the difference in motoneurone properties seen in young and adult animals could be the result of synaptic rearrangements within the spinal cord which in turn take place as a result of interaction of the motoneurones with their periphery. Since the subject of this thesis is the role of the interaction between motoneurones and muscles in maturation and development, it is relevant to consider next the postnatal development and properties of adult fast and slow muscles.

1.4.1. Characteristics of adult fast and slow muscles.

Mammals like rats and cats are born with immature muscles and nerves. The characteristics of these muscles change as the animals mature and the appropriate neural activity is imposed on them. They eventually fall into two extreme categories functionally and biochemically of slow and fast muscles.

Functionally, adult skeletal muscles can be divided according to their physiological properties into slow contracting and relaxing and fast contracting and relaxing. Biochemically, they can be divided into those with high levels of oxidative enzymes and those with high levels of glycolytic enzymes. Muscles containing a high level of oxidative enzymes can be either fast or slow contracting but those muscles which have a high level of glycolytic enzymes are usually fast contracting and relaxing. (Nachmias and Padykula, 1958; Dubowitz and Pearse, 1960a and b; Pette and Bucher, 1963; Dawson and Romanul, 1964). However, some studies have shown that the distinction between fast and slow
muscles is not so clear-cut since most muscles contain slow and fast contracting fibres (Gordon and Phillips, 1953; see also Vrbova, Gordon and Jones, 1978). In general, the distinction is valid for lower limb muscles of mammals however, and is thus relevant to the results presented in this thesis. Thus, for example, 80% of the soleus muscle in the rat consists of slow, oxidative muscle fibres and 20% of fast oxidative fibres whereas, the fast tibialis anterior muscle contains 40% fast glycolytic fibres, with the other 60% being largely fast oxidative interspersed with some slow oxidative muscle fibres.

The metabolic differences between slow and fast muscles are well-matched by their mechanical function and pattern of activation. Thus, fast muscles are capable of rapid tension development which is reflected in their high capacity for anaerobic glycolysis which in its turn is essential for rapid energy supply required by the contracting muscle. In general, fast muscle fibres are unable to maintain forces over long periods of time since they have to rebuild the glycolytic stores which they use up in producing large forces rapidly. Slow muscle fibres are unable to develop large tensions per surface area, but can maintain a given amount of force for long periods of time.

Dubowitz and Pearse (1960a and b) showed 2 distinct groups of muscle fibres on the basis of the enzyme activity they expressed. They have now been generally classified as follows. Slow fibres are called Type I and are characterised by high oxidative and low glycolytic enzyme activity. Type II fibres are generally fast, have high glycolytic and low oxidative enzyme activity. Engel (1962), has shown 2 distinct fibre types on the basis of myofibrillar ATPase (adenosine triphosphatase) activity. Muscle fibres with low myofibrillar ATPase have high levels of oxidative enzymes (Stein and Padykula, 1962;
Yellin and Guth, 1970; Barnard, Edgerton, Furukawa and Peter, 1971), while those with high myofibrillar ATPase activity have low oxidative capacity. A third type of fibre has been defined histochemically which has high ATPase activity as well as high oxidative capacity and been included amongst Type II fibres.

There are basic differences in the enzyme activity of the muscles which are directly related to the rate at which tension develops in slow and fast muscles (Gutmann et al., 1974). Although there is known to be a delay in the excitation-contraction coupling induced by calcium release from the associated structures to myofibrils, even in skinned slow tonic fibres calcium activation is slower than that seen in fast fibres subjected to similar treatment (Page, 1969; Constantin, 1974; Lannegren, 1975). The speed of shortening of a fibre is known to be related to the rate at which ATP (adenosine triphosphate) is split by actomyosin (see Close, 1972). The intrinsic speed of contraction has been shown to be proportional to the specific activity of myosin ATPase (Barany, 1967). Low and high specific myosin activity can be shown histochemically (Engel, 1962). Myosin light chains have since been implicated in regulating the myosin ATPase activity. The difference in structure of these light chains in slow and fast muscles has been thought to be responsible for the differences in their contractile properties (Weeds, 1969; Lowey and Risby, 1971; Syrovy, Gutmann and Melichna, 1972; Perry, 1974).

Morphologically, slow and fast muscles can be distinguished from each other by differences in fibre diameter, width of Z-lines, mitochondrial content, appearance of myofibrils and elaboration of the internal membrane system (Schiaffino et al., 1970; Luff and Atwood, 1971; Tomanek, Asmundson, Cooper and Barnard, 1973). Slow fibres of
the rat soleus muscle are smaller than those of fast EDL muscles. They are rich in mitochondria, have much thicker Z-lines and wide and less prominent M-lines. The sarcoplasmic reticulum (S-R) is also poorly developed in the soleus. The longitudinal tubules extending from the junctional cisternae are few and not as branched as are those of the EDL muscle (Eisenberg, Kuda and Peter, 1974). As a result, the myofibrils are not as regularly arranged as in the large fibres of the fast muscles in which the tubular system is more abundant. This last ultrastructural feature of fast muscles is seen in all fibres of the EDL muscle despite the fact that there are two different types - one rich in mitochondria and high in oxidative capacity and the other with few mitochondria and low in oxidative capacity. But the common ultrastructural features in the muscle may be related to its speed of contraction, since 95% of its muscle fibres are fast-contracting. The difference in the enzyme activity of the muscle fibres may be related only to their resistance to fatigue and their ATPase activity. But it has long been recognised that the characteristic properties of adult slow and fast muscles are modified by the type of neural activity they receive and that the two types of muscles are used preferentially in different types of movement.

1.4.2. Modification of muscle properties by neural activity and the formation of distinct motor units.

Fast largely glycolytic "pale" or "white" muscles (Ranvier, 1873) are generally used for brisk, quick movements requiring large forces that need not be maintained. Slow "red" and largely oxidative muscles on the other hand are used for maintaining steady forces such as those required in the maintenance of posture against gravity. For co-
ordinated movement to occur it is essential that the different types of motoneurones appropriately activate the different muscle fibres that they supply. Thus, in any given muscle, all the muscle fibres innervated by a single motoneurone form a functional unit or the motor unit. When a motoneurone is excited, all the muscle fibres in its domain are also excited. Thus the properties expressed by all the muscle fibres in one unit are the same and are imposed on them by their motoneurone. Sherrington (1904) saw the motoneurone and its axon as "the common final path" because it is via this route that all orders of the central nervous system are transmitted to the peripheral effector. Since different motoneurones are involved in different kinds of movement, it is essential that their properties match those of their effectors, the muscle fibres they innervate. Three types of motor units have been defined in the adult mammalian neuromuscular systems (for review, see Burke, 1981). The distinction of slow or fast muscle is dependent on which types of motor units predominate in any given muscle. Each motor unit contains muscle fibres under the influence of one motoneurone as defined earlier. Hence, all muscle fibres within a motor unit are homogeneous and display the same characteristics (see Edstrom and Kugelberg, 1968; Burke, 1981; Nemeth et al., 1981). The three categories of motor units correspond to the three main types of the motoneurones and muscle fibres within them. They are as follows.

Type I muscle fibres are of the slow oxidative variety (SO). They have slow speeds of contraction low glycolytic and intermediate to high levels of oxidative enzymes. They have low myosin ATPase activity and are resistant to fatigue.

Type II A and B muscle fibres are supplied by motoneurones that
have high frequency discharges, and have high thresholds of recruitment. They are also known to have large motoneurones (see Henneman, et al., Burke, 1981). Type II A are high in both oxidative and glycolytic activity, show high myosin ATPase activity and are fatigue resistant. They are otherwise called fast oxidative-glycolytic (FOG) or fast fatigue resistant (FFR) motor units (see, Burke 1981). Type II B are high in glycolytic activity and myosin ATPase and are fatiguable upon repeated stimulation. They are also known as fast fatiguable units (FF). How individual motoneurones achieve this matching with muscle fibres will be briefly considered in the next few paragraphs.

Denny-Brown (1929a,b) was the first to correlate the histological and contractile characteristics of slow and fast muscles of the cat to the activity imposed on them by their motoneurones. Recording the EMG (electromyogram) of slow soleus he found that motoneurones to the slow soleus muscle discharged at low rates for prolonged periods in response to the stretch reflex but that those to fast tibialis anterior (TA), fired in brief bursts at high rates. He also showed that motoneurones with low firing rates were recruited first and had low thresholds and that those with highest firing rates were recruited last and had high thresholds. Eccles et al., (1958) later confirmed these findings using intracellular recordings from spinal motoneurones. For co-ordinated movement to occur, smooth muscle contraction is essential and the slow contraction time of soleus muscles is well adjusted to the slow firing rate of motoneurones that supply them (Rack and Westbury, 1969).

Granit et al., (1956; 1957) classified motoneurones as "tonic" or "phasic" because of their behaviour after post tetanic potentiation of
muscle afferents. They found, as had Denny-Brown, that some motoneurones displayed sustained firing to stretch or other reflexes and others fired only a few high frequency spikes and were quiet (Granit et al., 1956; 1957). It was later shown that different motoneurones had differing thresholds of activation, because of their intrinsic properties (see Granit, 1972 for review). Muscles supplied by "fast" motoneurones show changes in their contractile properties after preceding activity imposed on them by their neurones by showing for example, post-tetanic potentiation and a slowing in their relaxation (Brown and Von Euler, 1938; Buller and Lewis, 1965). This enables them to maintain the same force even during the irregular slower, firing rates of their motoneurones during fatiguing exercise (Bigland-Ritchie et al., 1979; Hultman and Sjoholm, 1983). Thus, matching of motoneurone and muscle properties is achieved and results in a functional motor unit. "Slow" motoneurones have been shown to receive stronger monosynaptic input than fast ones. Since their afterhyperpolarization is known to be longer, it has been suggested that their firing rate is controlled by this parameter (Eccles et al., 1958). As has been already discussed, slow muscles reflect these properties in their mechanical function (Rack and Westbury, 1969). "Slow" motoneurones have been shown to vary their firing rate less than "fast" ones. They increase the amount of force produced by the muscle by recruiting more motor units rather than by increasing their firing rates (Freund, 1983). Consequently, slow muscles do not show a great change in their contractile properties in response to previous activation and only show very small post-tetanic modification.

That motoneurones can permanently modify the properties of the muscles they innervate has been clearly shown by the classic cross-
reinnervation experiments of Buller, Eccles and Eccles (1960b). They found that innervating the slow soleus muscle with the nerve of the fast flexor digitorum longus (FDL) muscle made the soleus muscle into a fast contracting and relaxing muscle. The opposite was the case in the FDL muscle which was reinnervated by the original nerve to the slow soleus muscle. Furthermore, this transformation occurred whether "cross-innervation" was performed in adult or young cats and only failed to happen if motoneurone activity was reduced by spinal cord transection and deafferentation (Buller et al., 1960b).

Buller et al. (1960b) considered two possibilities to explain these findings. Firstly, the factor determining the contractile properties of a given muscle could be the frequency or aggregate activity of its motoneurone. Secondly, the two different types of motoneurone may release their individual "trophic" factors that brought about the change seen in muscles.

Although the first explanation was the simpler one and accommodated results of previous findings that activity patterns of motor nerves remain unaltered even when they were transposed to different muscles (Sperry, 1945), they preferred the second alternative. This was because "trophic" influences of motoneurones independent of their activity had been thought to be responsible for changes seen in denervated muscles in several previous studies (Tower, 1937a,b; Luco and Eyzaguirre, 1955; Miledi, 1960b).

But subsequent evidence relating to changes in activity patterns of normally innervated muscles has pointed to the first alternative as being the likely explanation for the influence of the motoneurone on the properties of the muscle fibres it innervates. When fast muscles
have been electrically stimulated via their motor nerves at low frequencies, they acquire the characteristics that resemble those of slow muscles over a period of time (Vrbová, 1966; Salmons and Vrbová, 1969). Thus, it is clear that the impulse pattern from the motoneurone is the primary determinant of the contractile properties of adult skeletal muscles (Pette et al., 1973; Lomo et al., 1974; Salmons and Sreter, 1976; Buller and Pope, 1977; Pette and Vrbová, 1985; see also Vrbová and Wareham, 1976).

Histochemical profiles of muscles after cross-innervation or chronic electrical stimulation show a similar trend as that seen in their contractile properties. Slow soleus muscle fibres change from homogeneously high aerobic type to anaerobic ones seen usually in fast muscles as a result of cross-innervation (Romanul and Van der Meulen, 1967). Similarly, myosin ATPase activity which is lower in slow than in fast muscles (Barany, 1967), is transformed to that resembling a fast muscle after cross-innervation (Barany and Close, 1971).

After chronic electrical stimulation of fast muscles these anaerobic muscles become aerobic ones (Pette et al., 1973). Changes in sarcoplasmic reticulum (S-R) and calcium binding are also seen (Ramirez and Pette, 1974). Finally, myosin light chains in fast muscles resemble those of slow muscles as a result of chronic electrical stimulation at low frequencies (Salmons and Sreter, 1976; see also Pette and Vrbová, 1985; Pette and Staron, 1988).

Thus, at least for adult animals, motoneurone activity is very important for the maintenance of normal muscle function. The dependence of muscle on motoneurone activity is already apparent during maturation and development and this will be considered next.
1.4.3. Postnatal development of fast and slow muscles.

Future fast and slow muscles of newborn mammals are initially slowly contracting and relaxing. There is however a difference in the rate of increase in the speeds of contraction of fast and slow rat muscles from birth to about three weeks of age (Close, 1964; Gutmann et al., 1974). The most important differences between adult slow and fast muscles develop during this postnatal period. Some evidence exists that at birth, oxidative enzyme activity is high in all muscles (Dubowitz, 1965) and that this changes into the relevant activity pattern seen in adult muscles by the first 21 days of life. Consistent with the change in enzyme activity that occurs as a consequence of development, is the relative fatigue resistance of neonatal muscles at first and which gradually decreases only in fast muscles (see Hammarberg and Kellerth, 1975c).

The troponin complex changes also during the course of development of slow and fast muscles (see Dhoot, 1986). This seems to be regulated by the motoneurone as long as interaction with the target is maintained. Sarcoplasmic reticulum changes occur and can be seen morphologically and biochemically. The S-R increases in surface area which increases its capacity to pump calcium (see Luff and Atwood, 1971; Kelly, 1980). This leads to a decrease in the duration of the active state of the muscle, so that the time during which the interaction between contractile proteins takes place is shorter (see Close, 1964; 1972). Calcium uptake in fragments of S-R in neonatal rat EDL muscles has been studied by Drachman and Johnston (1973). They have found that this rapidly accelerates after birth and is correlated with an increase in speed of contraction and relaxation of the muscle
Developmental changes in speed of contraction of fast and slow muscles result because of maturation of the changes occurring in different compartments of each muscle fibre (Close, 1972; Vrbová, 1980). Two factors underlie these changes. Firstly, the duration of the active state is related to the ability of the S-R to sequester the "activator" calcium. Secondly, the intrinsic speed of shortening of muscle fibres is determined by the type of contractile proteins being present. The intracellular mechanisms involved in the differentiation of these parameters is still under investigation and no consensus has been reached as yet especially with regard to the types and role of myosin isoenzymes within neonatal muscles.

Two distinct types of myosin in isoenzymic forms can be detected to be synthesized by slow and fast muscles and these have been identified by the differences in their structure (Jolesz and Sreter, 1981). Previous evidence has indicated that neonatal muscles seem to have both slow and fast forms within the same muscle fibre although the "fast" form predominates (Rubinstein and Kelly, 1978; Kelly and Rubinstein, 1980; see also Rubinstein et al., 1988).

Some other workers have suggested a third isoenzymic form of myosin which they call embryonic myosin (Trayer et al., 1968; Whalen et al., 1981). Whalen et al. (1981) have suggested that myosin isoenzymic forms develop sequentially. Firstly, the embryonic form is seen in primary myotubes and only later do the other forms of enzymes appear. However, a controversy surrounds the definition and types and expression of myosin isoenzymes in neonatal muscles and are beyond the scope of the present work. In as much as the expression of different myosins does concern us here, it would be relevant to point out that
some indirect evidence does exist to show that the expression of myosins, either fast or slow, is regulated by the activity imposed on the muscle by its motoneurone (Gutmann et al., 1974; Rubinstein and Kelly, 1978; Margreth et al., 1980). Several studies on reinnervation have shown that the actual process of innervation appears to be non-selective in both young and adult muscles (Jones et al., 1987; Lowrie et al., 1988). The determining factor at least for young muscles for the expression and development of their normal characteristics seems to be their readiness to accept the activity imposed on them by their motoneurones. This will be considered in the next section.

Part II.

1.5.1 Effects of disconnection of adult motoneurones and muscles.

Adult skeletal muscles depend on their motor innervation for maintenance. However, they can also survive temporary denervation provided that reinnervation is allowed to proceed unhindered. Thus, after sciatic nerve crush in adult rabbits or rats, muscles recover virtually completely their weights and tensions upon reinnervation (Gutmann and Young, 1944; Beránek, Hník and Vrbová, 1957; Lowrie et al., 1982). Also, when these muscles become reinnervated by their own nerves, the axons are usually guided to their original end-plate sites and neuromuscular contacts are restored (Gutmann and Young, 1944; Miledi, 1960b).

Effects of nerve crush or axotomy in the peripheral nervous system of adult animals causes a series of changes of properties of motoneurones, their cell-bodies, axons, the neuromuscular junction and the muscle they innervate. However, all these changes are reversed upon reinnervation. Some effects of peripheral nerve injury on the
cell bodies and axons of neurones will be considered next.

a) Immediate Effects of axotomy: Wallerian degeneration in the distal stump.

Although Cajal (1928) was the first to visualise degenerating axons, Holmes, (1915) was the first to study axons that were injured and to recognize that they swell after injury. An explanation for this "bulging" of axons seen after injury came with the discovery of axoplasmic transport (Weiss, et al., 1948). It has been postulated that axoplasmic transport continually provides the flow of macromolecules from the perikaryon to the synaptic terminal and by doing so is fundamentally important to axonal regeneration (Lasek, 1982).

Axons have little or no capacity for protein or lipid synthesis if they are isolated, distal stumps because they contain few ribosomes (Zelenà, 1972). The cell body is the main site of macromolecular synthesis and axons depend on transport systems to supply them with the required materials synthesized there (Lasek & Black 1977; see Gordon, 1983). Axons physically separated from the cell body die and the degeneration of these distal stumps is called Wallerian degeneration, first described by Waller (1850). He showed that the cell body was essential for axon survival and attachment just to the target was not an adequate substitute. Cajal (1928), postulated that the degeneration occurred simultaneously along the entire isolated stump. However, more recent evidence shows that it proceeds in a proximo-distal manner (Sunderland, 1978; see Gordon, 1983). In the rat, for example, after axotomy, neuromuscular failure is delayed by 2 hours for every additional centimetre of distal nerve stump (Miledi
and Slater, 1968; 1970). In addition, direct evidence for the longitudinal spread of degeneration came from histological studies of the distal nerve stump (see Gordon, 1983) which showed that the number of degenerating axons was highest closest to the site of the injury and decreased along the length of the stump.

Short-term survival of distal stumps can still occur because nutrients are transported by axoplasmic transport until they are depleted (Lubinska, et al., 1971). Glial cells have also been shown to provide some of the proteins required to support (Lasek et al., 1977), but these are not enough for the maintenance of mammalian axons in the absence of their cell bodies or without axonal transport.

The bulk of these proteins are transported by slow axoplasmic transport of 1 to 5 mm per day from the cell body down the axon. Some of these are tubulin, actin and the subunits of neurofilaments (Hoffman & Lasek, 1975; Willard, 1977). The rapid phase of axoplasmic transport is 2 orders of magnitude faster than this and is 400 mm per day in mammalian peripheral axons (see Gordon, 1983 for review). Particulate material such as membranous organelles for normal membrane turnover, transmitter synthesis and secretion and axonal metabolism are transported by the fast phase (see Gordon, 1983; McQuarrie, 1988).

Fast transport can continue in isolated segments as can neuromuscular transmission. However, the latter will only continue until the supply of neurotransmitter is depleted and fails quicker if terminals are activated and chemical transmission goes before impulse conduction (see Gordon, 1983). All these changes in the distal stumps of severed axons and the proximo-distal direction of depletion of nutrients varying with transport rates and usage of materials points to the fact that degeneration spreads in an anterograde manner from
the site of the injury to the terminal (see Gordon, 1983).

The last stages of Wallerian degeneration involve dissolution and disorganization of neurotubules and neurofilaments with the disintegration of the axons, their phagocytosis and the intense proliferation of Schwann cells (see review by Gutmann, 1958; Sunderland, 1978).

The final stage of degeneration of the distal stump occurs because of the lack of turnover of nutrients and the death of mitochondria which are not replaced but accumulate at the injured end (Webster, 1962). Failure of oxidative phosphorylation would lead to loss of membrane potential and disruption of chemical and electrical gradients. Additionally, the accumulation of calcium ions in the axolemma causes the rapid depolymerization of microtubules and may also be involved in the activation of hydrolytic enzymes. However, distal stumps can survive if these final stages are avoided. The evidence comes from experiments on peripheral stumps in culture. If all the nutrients are artificially supplied, these peripheral stumps do not die but even grow and fuse again with the growing proximal stump of the parent neuroblast (Boeke, 1950).

b) The chromatolytic response of the axotomised neurone.

Nissl (1892) was the first to describe morphological alterations in the soma of axotomised neurones. The reaction of the soma of the motoneurone to axon injury has been reviewed by Lieberman (1971; 1974). If the re-establishment of connections with the muscles is prevented, axosomatic boutons or dendritic branchings are lost (see Lieberman, 1974) and not restored. The cytoplasm of the soma undergoes several changes too. Chromatolysis may persist for several months
after the injury if it is an axotomy and not a nerve crush (Barr & Hamilton 1948; Lieberman, 1974; see also Sears, 1987). However, motoneurones persist almost indefinitely even if they are prevented from reestablishing connections with the target (Barr & Hamilton, 1948). But even these persistent motoneurones eventually degenerate and perish if they are prevented from reaching their targets.

The morphological changes seen in the axotomised soma are the swelling and migration of the nucleus to an eccentric position, and an increase in nuclear and nucleolar size. These changes refer particularly to the apparent disappearance of prominent basophilic staining Nissl granules which are ribosome clusters and ordered arrays of rough endoplasmic reticulum. These can no longer be visualised when they become disorganised, freeing polyribosomes and even ribonucleotides into the cytoplasm. The disorganization of ribosome clusters indicates morphological changes which are associated with protein synthesis. Thus, the chromatolytic response is the anabolic response of an injured neurone. Evidence from studies on RNA and protein content in axotomised neurones supports this premise. Thus, several studies have shown that there is decreased DNA repression, an increase in RNA synthesis with the transfer of RNA from nucleus to cytoplasm and an increase in the cellular protein content. The concentration of enzymes required for RNA synthesis have also been shown to increase (for review see Gordon, 1983). The changes in RNA protein synthesis coincide with the maximal chromatolytic response observed histologically (Cragg, 1970; see also Johnson et al., 1985). It has also been observed that these chromatolytic changes were more pronounced the closer the axotomy was to the cell-body (see Gordon, 1983). It was thought therefore that chromatolysis was the metabolic
response of the cell-body to reconstitute the loss of the length of the distal stump. However, several injured neurones for example, nodose ganglia in the rat, do not show a chromatolytic response (see Lieberman, 1971).

However, evidence indicates that the changes in the protein content and RNA mentioned above are later manifestations of an early turnover and increase in RNA metabolism. These early changes are shown to occur within hours of the injury rather than days (Gunning et al., 1977; Kaye et al., 1977 Burrel et al., 1978;). The signal for the change seems to be very rapid and alterations of gene expression have been thought to be involved but these are still under speculation. For instance, phospholipid changes occur within half an hour of axotomy, and this suggests that the signal must be transported at 140mm per hour, a rate which is much faster than normal fast axonal transport (see Dziegielewska et al., 1980).

Several authors (Cragg, 1970; see Gordon,1983 ), thought that chromatolysis was a response to injury and was not the regenerative response of axotomised motoneurones. This was because chromatolysis involves nuclear and ribosomal function, sequestration of intracellular organelles in membrane bound vacuoles with ultimate degeneration in the lysosomes (see Lieberman, 1971).

What then is the function of chromatolysis? Watson (1970) found that the increase in RNA associated with chromatolysis continued even when functional connections were re-established. He found a 2nd burst of RNA synthesis after nerve crush of already axotomised hypoglossal neurones. He interpreted this burst as a transformation of the axotomised neurones from a "resting, non-transmitting mode" to a
second "seeking mode". It has been suggested that this second burst of RNA synthesis may account for the enhancement of regeneration seen after two consecutive lesions (see Gutmann, 1942; McQuarrie and Grafstein, 1973). The final RNA burst that occurred with the re-establishment of functional connections was thought to be related to an increase in the synthesis of neurotransmitters and their precursors to the regenerating nerve terminal so that the regenerating axon was prepared for the change from the "growing, seeking" mode to the "transmitting" mode (see Gordon, 1983).

It appears from several studies that chromatolytic changes occur in the neurone even when the axon is left intact but is poisoned with botulinum toxin (Watson, 1969) and that as a result of this, sprouting occurs during the course of muscle paralysis (Duchen & Strich, 1968). The time course of recovery of functional neuromuscular transmission after such poisoning has been shown to be similar to that of muscles reinnervated by regenerated axons after axotomy (Tonge, 1974a,b).

The above evidence is in agreement with a recent study on the target dependence of Nissl body ultrastructure of cat thoracic motoneurones for its maintenance (Johnson et al., 1985). The study used reversible (nerve crush) or "chronic" axotomy (transection of the axon with proximal ligation) of external intercostal nerves to study the changes in Nissl body ultrastructure of their motoneurones as a measure of altered protein synthesis. Under the electron microscope they found that cell bodies of axons allowed to reinnervate their targets after nerve crush returned to the normal ordered organization of the Nissl body structure between 64 and 208 days after the injury. The motoneurones that had suffered "chronic" axotomy and were prevented from reinnervation showed very little organization of their
ultrastructure. To ensure that this was due to non contact with the intercostal muscles, the severed axons which had formed a neuroma were resectioned 49 days later and allowed to regenerate into a newly sectioned internal intercostal nerve. 64 days later, some of the external intercostal motoneurones of these redirected regenerated axons showed that normal Nissl bodies had formed in them whereas none were seen in control chronically axotomised motoneurones in the adjacent but one segment. The authors conclude from these results that axonal elongation is not a sufficient factor for the regeneration of Nissl bodies since even after reversible axotomy, Nissl bodies are still absent prior to reinnervation and 33 days after the injury. They therefore suggest that the signal for Nissl body regeneration does not come from Schwann cells or peripheral nerve sheaths (Lundborg et al., 1982; Politis et al., 1982). Normal Nissl body structure appears to be dependent on either the presynaptic motor nerve terminal or the muscle. Thus, it may depend on the transport of some protein from the target or from the cell body which would only be activated by contact with the target (Willard and Skene, 1982; see Ingoglia et al., 1984). On the other hand it may depend on some independently acting muscle-derived signal (see Lieberman, 1974, for "trophic" influences on the cell body). The signal for growth or regeneration may depend upon the basic cytoskeletal proteins and their role in the expression and synthesis of the RNA within the cell body and this will be considered briefly.

c) The dependence of the axon on the cell body for successful regeneration to occur.

Axonal regeneration has been thought to depend on the re-establishment of the cytoskeleton. The major components of this axonal
cytoskeleton are neurofilaments, microtubules and cross-linking structures (Hirokawa, 1982). Neurofilaments consist of three distinct protein subunits (Hoffman and Lasek, 1975). The main subunit of microtubules is tubulin. It has been shown that tubulin and neurofilament proteins are directly involved in the process of growth and regeneration in the axon (Lasek and Hoffman, 1976) and that they are assembled in the soma because the axon is devoid of ribosomes. They are transported via slow axonal transport at 1-2 mm per day (Hoffman and Lasek, 1975). Neurofilament proteins are assembled in the cell body shortly after synthesis and undergo axonal transport in the form of neurofilament polymers (Black and Smith, 1988); axons do not contain unassembled neurofilament protein (Morris and Lasek, 1982). On the other hand, it is likely that unassembled tubulin and assembled microtubules are transported down the axon (Brady et al., 1984; see also Hoffman, 1988). The transport of unassembled tubulin means that there is a potential for the assembly of microtubules in the distal axon.

In general, tubulin has been implicated in the mechanism of axonal elongation (Yamada, et al., 1971) whereas neurofilaments seem to be involved in radial growth (see Hoffman, 1988). Thus, it is not surprising that tubulin is expressed in high levels in developing or regenerating nerves since microtubules are the main cytoskeletal elements seen in them (Berthold, 1978). It has been suggested that it is this ability of microtubules to undergo assembly and disassembly that may contribute to the plasticity of developing or already regenerating adult axons (Lasek, 1981; see section b) above). In addition, the rate of regeneration (and therefore of axonal elongation) may be limited by the delivery of tubulin by slow axonal
transport (Lasek & Hoffman, 1976; Wujek & Lasek, 1983).

Neurofilaments are the intrinsic determinants of axonal calibre in myelinated nerves (Friede & Samorajski, 1970; Weiss & Mayr, 1971; Berthold, 1978; Hoffman et al., 1984). It is not surprising that developing and regenerating neurones contain few neurofilaments but are rich in microtubules. (Peters and Vaughn, 1967; Berthold, 1978). The decrease in axon calibre of the proximal stump which occurs as a result of axotomy (Greenman, 1913) correlates with reduced neurofilament protein gene expression (Hoffman et al., 1987; Hoffman, 1988). This axonal atrophy is associated with a reduction of content of neurofilament proteins in the axon (Hoffman et al., 1984) and in its transport in the proximal stump (Hoffman et al., 1985b). This atrophy is seen first proximally and spreads down the axon stump at the rate at which neurofilament proteins would be transported and has thus been called somatofugal axonal atrophy (Hoffman et al., 1987). Since axonal calibre is the most important determinant of conduction velocity, (Hursh, 1939) somatofugal atrophy is associated with reduced axonal conduction velocity in the proximal stump of regenerating nerves (Cragg and Thomas, 1961; Gillespie and Stein, 1983). Once reinnervation is allowed to occur, neurofilament expression (Hoffman et al., 1984) and axonal calibre (Kuno et al., 1974) recover to pre-axotomy levels.

From the above cited evidence it is obvious that disruption of axonal transport would be debilitating for the axon unless its cytostructural elements are restored.

d) Other effects of axotomy on neurones and axons.

Axonal injury reduces the amplitude of the monosynaptic excitatory
post synaptic potential (Eccles et al., 1959; Kuno et al., 1970; Mendell, 1984). That this effect is reversed upon reinnervation has been shown. Both muscle and nerve action potentials show a progressive fall in amplitude upon nerve transection in adult animals. Latencies of compound action potentials have been shown to increase in the proximal stump after injury and their amplitude to decrease and that these properties do not recover until peripheral connections are re-established (Davis et al., 1978; Orcozo et al., 1985). The recovery of these properties can be inferred from nerve conduction velocity studies of Cragg and Thomas, (1961; 1964; also Thomas, 1964). These workers found that conduction velocities of axotomised nerves were reduced by 10 to 25% at the time of the lesion but were reversed to normal values upon target reinnervation. The same was also found by Gordon and Stein (1982a). This has also been shown in studies on human nerve injuries, where conduction velocities of injured axons returned to 90% those of normal, 2 years after the injury (Donoso et al., 1979).

The change in fibre diameter of the axon after axotomy was first shown by Greenman (1913) using histological techniques. The fibre size of the central stump has been shown to decrease after injury and atrophy of axons is greatest if reinnervation of peripheral end organs is prevented. Normal peripheral nerves consist of mixed myelinated and unmyelinated fibres. The axon and myelin thickness of these normal fibres is generally bimodally distributed (Boyd and Davey, 1968). However, this bimodality is lost after axotomy and fibre diameter distribution becomes unimodal (Gutmann & Sanders, 1943; Weiss et al., 1945). As has been discussed above (see section (c)), the progression of atrophy in the axon is similar to the rate of transport of
neurofilament triplet proteins and the decline in axon diameter after axotomy may be directly related to their decreased axonal transport (see Hoffman, 1988).

Sensory axons continue to atrophy after motor axons have ceased to do so (Hoffer et al., 1979). But it is the fastest conducting axons that degenerate most rapidly (Milner and Stein, 1981) and it is their sprouts which mature most rapidly (Devor and Govrin-Lippman, 1979a). In addition, severed motor axons continue to show activity indefinitely despite no peripheral connections but sensory fibres remained quiet (Hoffer, et al., 1979; Milner and Stein, 1981).

Myelin thickness has been shown to be unaffected by axotomy and is resistant to atrophy. Ultrastructural studies have shown that myelin thickness remains constant as actual axonal diameter decreases (Gillespie and Stein, 1982; see also Gordon, 1983). These are consistent with earlier studies using light microscopy which showed that there was a decrease in axonal diameter rather than the total fibre diameter (axon plus myelin) (Sanders, 1948; Cragg and Thomas, 1961).

Measurements of conduction velocity have always used the total fibre diameter of degenerating and regenerating nerves (Hursh, 1939; see also Gordon, 1983) and caused some controversy (see Cragg and Thomas 1961). However, most early studies have shown conduction velocity decreased in the central stump after peripheral nerve section (Gutmann and Holubar, 1951; Eccles et al., 1959; Kiraly and Krnjevic, 1959; Cragg and Thomas, 1961). Gillespie and Stein (1982) have shown that conduction velocity always varied with axon diameter and not the total fibre diameter. It has also been found that the nodes of Ranvier
remain unaffected after nerve lesions as do internodes (see Cragg and Thomas, 1961).

Neuromuscular transmission has been shown to fail 8 to 10 hours after nerve section in the rat (Miledi and Slater, 1970). That there is a direct relationship between neuromuscular transmission failure and the site of the lesion has been shown by the fact that with each additional centimetre on the distal stump neuromuscular transmission was delayed by 45 minutes. Once reinnervation of the target occurs however, the motoneurone and its axon recovers all its normal functional properties (see Miller, 1987).

e) Regeneration and reinnervation of target organs.

Axotomised nerves have been shown to grow along interfaces provided by pioneer fibres or locally derived cells (Harrison, 1935; Weiss, 1950). The first stage is growth, the second is development and maturation followed by interaction with the target (see Gordon, 1983). Regenerating axons are thought to recapitulate this two stage growth process. It is suggested that their growth and dimensions are determined by the parent fibres and the direction of their growth is provided by the vacated Schwann tubes in the distal nerve stump. Although the specific guidance cues of Schwann cells may be lost in regenerating adult axons (unlike developing axons see Landmesser, 1986), it is clear that they play an important supportive role in guiding the regenerating axons to their targets (Keynes, 1987). The regenerating axons can be maintained indefinitely in this first stage by the adult cell body (Davis et al., 1978; see also Johnson et al., 1985). However, for the second stage of regeneration to occur, interactions with the target are essential. For example if nerves are crushed or ligated to the fascia they show different patterns of
regeneration (Weiss et al., 1945; Aitken, et al., 1947). In the former condition, the axons regenerate and sprout into the distal stump but in the latter they only get as far as the obstruction and no sprouts are seen.

A recent study has shown that upon reinnervation, the axons of regenerated common lateral gastrocnemius soleus nerve to the fast gastrocnemius and slow soleus showed no specificity of returning to their original muscles so that the same proportion of fast and slow motor units were seen in both muscles (Gillespie et al., 1986; Gordon, et al., 1988). However, muscle fibres were re-specified in terms of their histochemical properties, contractile speeds and force outputs. The net result is neuromuscular plasticity and the re-establishment of motor units with force outputs that vary sufficiently for the orderly recruitment of motor units required in movement (see Gordon, et al., 1988).

The situation is not the same if peripheral nerve injury is inflicted early in life. Although reinnervation occurs it leaves permanent and lasting effects on both motoneurones and their muscles. The next section will consider the effects of temporary disruption of interaction between motoneurones and muscles during development of the neuromuscular system.

1.5.2. Effects of peripheral nerve injury in early postnatal life on the developing neuromuscular system.

From the work reviewed in previous sections, it can be seen that motoneurones and muscles are functionally interdependent. The effects of deprivation of interaction between nerves and muscles has a detrimental effect on the normal functioning of the neuromuscular
system as it results in muscle paralysis and alteration in motoneurone properties. However, in adult animals these effects are reversed if interaction between them is restored by reinnervation (see Gordon, 1983). Disconnection between nerves and muscles during embryogenesis and after naturally occurring cell death has ceased, results in the inadequate development of muscle fibres and often the death of some of their motoneurones. This critical dependence on contact between motoneurones and muscle fibres continues into early postnatal life (see Vrbova et al., 1985).

The severity of the effects of temporary denervation on muscles and motoneurones during early postnatal life decreases as the age at which the injury is inflicted increases. This has been demonstrated in several species and using different neuromuscular systems as experimental models.

a) Effects of axotomy or nerve crush on neonatal motoneurones.

Several workers have investigated the effects of sciatic nerve injury at birth and have found that many motoneurones are lost as a result of such an injury (Romanes, 1946; Beuker and Meyers, 1951; Zelena and Hnik, 1963; McArdle and Sansone, 1977; Schmalbruch, 1984; Lowrie et al., 1987). This vulnerability of motoneurones to nerve injury decreases progressively with age (Romanes, 1946; Lowrie et al., 1982; 1984; 1987) so that by adulthood no loss of motoneurones occurs as a result of temporary denervation. This has been demonstrated in several other species such as guinea pig spinal neurones (Hess, 1956; 1957) and hamsters (La Velle and La Velle, 1959; La Velle, 1964). In addition, if these young motoneurones are prevented from reinnervating their targets, for more than two weeks they degenerate and die (see
Kashihara et al., 1987). However, no motoneurone loss has been shown to occur if the injury is made very close to the target muscle and reinnervation is left to proceed unhindered (Brown et al., 1976; Kashihara et al., 1987).

In rats, after sciatic nerve crush at birth the proportional loss of motoneurones was similar in slow and fast muscles (see Lowrie et al., 1987). In addition, even when no motoneurones were lost after a sciatic nerve crush at 5 days, they remained permanently smaller than those from normal age and weight matched control animals (Lowrie et al., 1982).

No direct evidence exists as to the effects of disconnection of electrophysiological properties of young motoneurones from their targets. However, this may be inferred from studies on adult motoneurones. It has been shown that adult motoneurones require functional contact with their targets (Czeh et al., 1977) and if they are denied it, they revert to the electrophysiological properties of immature motoneurones (Kuno et al., 1974; Huizar et al., 1975). In addition, the evidence on the electrophysiological properties of neonatal motoneurones (see Section 1.3.2) shows that they are as yet immature and activated by general stimulation.

Indirect evidence exists from the studies of Navarrete and Vrbova (1984) on the EMG of young fast and slow muscles that show a permanent alteration of their activity pattern as a result of neonatal sciatic nerve injury. If the muscle is a mirror of the activity imposed on it by its motoneurone then the activity of young motoneurones is certainly altered by a nerve injury early in postnatal life (see Vrbova et al., 1985). The permanent increase in the EMG activity of reinnervated muscles after a neonatal nerve injury has been shown to
be more apparent in fast extensor digitorum longus (EDL) muscles than in slow soleus muscles (see Navarrete and Vrbová, 1984). This correlates well with the idea proposed by Vrbová et al., (1985) that young muscles and motoneurones need to interact continuously to develop appropriately matching properties and that a temporary disruption of this interaction would lead to a mismatch between the two. In addition, Navarrete and Vrbová (1983) have also shown that the development of adult activity patterns in muscles occurred only after birth within the first three weeks of postnatal life. Thus, the characteristic tonic low firing patterns at low frequencies of slow Soleus muscles and the phasic high frequency firing patterns of fast EDL muscles develop gradually by the third week of postnatal life.

b) Effects of axotomy in neonatal animals on the development of muscle properties.

Motoneurone loss as a result of sciatic nerve injury at birth causes degenerative changes in the muscles upon their reinnervation. These are expressed as loss of muscle weight and fibres and a reduction in the maximum tetanic tension produced by them (Zelená and Hnik, 1963; Lowrie et al., 1987). Zelena and Hnik (1963) also showed that after sciatic nerve crush at birth, the reinnervated soleus muscle had fewer but larger muscle fibres than normal muscles. Motor fibre numbers were reduced to 50% that of contralateral controls and their mean fibre diameter was 75% that of normal. Motor unit size too remained permanently small compared to normal despite the fact that the size of reinnervated muscle fibres was larger. Furthermore, evidence from later studies has shown that reinnervated soleus muscles were faster than normal age-matched controls whereas reinnervated fast
muscles remained significantly slower than normal (Lowrie et al., 1987). In addition, the number of remaining motoneurones to both fast and slow muscles was similar as a result of nerve crush at birth, 42% and 31% of the normal motoneurone pool to the fast tibialis anterior (TA) and extensor digitorum longus (EDL) and slow soleus muscles respectively. However, there was a greater discrepancy between the recovery of strength and motoneurone number of the former (just 10% to 17% of normal age matched control muscles) whereas this was not the case for the slow soleus muscle (Lowrie et al., 1987).

Other muscle properties that were affected after nerve crush at birth were twitch contraction and relaxation times. Thus, soleus muscles which normally contract and relax slowly, upon reinnervation after nerve injury at birth, had significantly faster than normal contraction and relaxation times. The reverse has been found to be the case with the fast muscles, TA and EDL (see Lowrie et al., 1987).

As has already been stated, evidence has shown that the motoneurone's susceptibility to death as a result of injury to its axon is reduced as its age increases (Romanes, 1946; Hess, 1956; 1957; La Velle and La Velle, 1959; La Velle, 1964; Lowrie et al., 1982; 1984; 1987).

In rats, after sciatic nerve injury at 5 days no motoneurone loss is seen but reinnervated fast muscles such as TA and EDL remain permanently impaired (Lowrie et al., 1982; 1984; 1987). Their weights and tensions remain at 50% of contralateral control or normal muscles. Surprisingly, reinnervated soleus muscles recover to 80% of their contralateral controls. Since no motoneurone death is seen to occur, the selective impairment of the reinnervated fast muscles cannot be attributed to this.
It is as yet unclear why fast muscles lose many muscle fibres as a result of temporary disconnection from their motoneurones early in postnatal life. This could be due to changes in motoneurones that make it difficult to maintain a normal sized peripheral field (see Subramaniam Krishnan et al., 1985; Albani et al., 1989). Or, it may be due to impairment in muscle development. Evidence suggests that following nerve crush the isolation of the motoneurone from its target interferes with the development and maturation of both, just at the time when interaction between them is crucial (see Vrbova et al., 1985). The loss of muscle fibres in fast muscles after sciatic nerve crush at 5 days found by Lowrie et al., (1982) occurred not upon denervation but reinnervation (Lowrie and Vrbová, 1984). In addition, the metabolic properties of these reinnervated fast muscles too were altered. Compatible with an increase in their functional activity (Navarrete and Vrbová, 1984), they also showed an increase in fatigue resistance and oxidative enzymes when stained with succinic dehydrogenase (Lowrie et al., 1982). This contrasted with normal fast muscles which were very fatiguable and low in oxidative enzyme activity as shown by the use of succinic dehydrogenase (SDH) (see also sections 1.4.1 to 1.4.3). Thus, upon reinnervation, no large pale fibres were seen in fast muscles and the remaining fibres were regrouped into clusters and the mosaic pattern seen in normal muscles was lost. Since large pale fibres are glycolytic and contain fast myosin (see section 1.4.1), it follows that the pattern seen upon reinnervation of fast muscles was at the expense of these fast fibres. Using an antibody against slow myosin, Lowrie et al., (1988) found that 3 months after the initial injury, most of the fibres in
reinnervated EDL muscles contained slow myosin. At this stage the muscles also stained uniformly with SDH and no pale fibres were seen. The suggestion from this study was therefore, that the reinnervated muscle fibres that remained were gradually being converted to slow ones since earlier studies had shown that the sequential appearance of either fast or slow myosin during normal development and adult reinnervation happened at the expense of the other. So, as one myosin was synthesized the other was degraded (Dhoot, 1986). Since the normal number of slow muscle fibres was found to be preserved in reinnervated fast muscles (Lowrie et al., 1988), and no motoneurones were lost (Lowrie et al., 1982), yet 50% of the muscle fibres were, it followed that the size of some motor units must have decreased. This decrease in motor unit size was perhaps due to the selective loss of fast muscle fibres. Thus, the results of the study presented above (Albani et al., 1988) were in agreement with evidence from adult mixed muscles in which slow motor units were reported to have had a selective advantage during reinnervation (Lewis et al., 1982; Foehring et al., 1986).

The evidence points to the fact that fast muscles appear to be more vulnerable to neonatal nerve injury than do slow muscles. Although the reasons for this still remain unclear, the main outcome of previous research discussed here suggests that fast muscles require constant interaction with their motoneurones to achieve properties that will match them.

The aim of the experiments described in this thesis was to investigate further the mismatch between young muscles and their motoneurones.

In the first part of the thesis, results of experiments following
the early stages of recovery of fast and slow muscles after sciatic nerve crush at 5 days are described. A comparison between the patterns of reinnervation of slow Soleus and fast EDL and TA muscles was made in order to find out if there was a difference in the selectively improved recovery of the former.

Chapter 3 describes effects of varying the time of separation between the nerve and its muscle. This was done in order to find out if shorter periods of separation would reduce the mismatch between the muscles and their motoneurones.

Chapter 4 describes effects of unilateral nerve injury in young animals on the development of their reflex activity.

Finally, Chapter 5 will discuss the results of experiments that were performed in attempt to improve the chances of survival of motoneurones after sciatic nerve crush at birth.
CHAPTER 2.

The initial stages of muscle recovery after sciatic nerve crush in 5-day old rats. Effects on Tibialis Anterior, Extensor Digitorum Longus and Soleus muscles.

2.1 Introduction.

The physiological properties of mammalian skeletal muscle fibres are known to be matched to those of the motoneurones that innervate them. Muscle fibres activated by low rates of firing by their motoneurones contract and relax slowly, while muscle fibres activated at fast rates of firing contract and relax rapidly (see Vrbova, Gordon and Jones, 1978 for a review).

Thus, the mechanical properties of adult muscles are matched to the physiological characteristics of their motoneurones (see Vrbova et al., 1985). This matching develops as the neuromuscular system matures. Muscle cells are initially formed and develop independently from their motoneurones (see Chapter 1: General Introduction), and it is only later when motoneurones establish contact with them that they become dependent on their motoneurones. As development proceeds the muscles together with the motoneurones that supply them become functional motor units.

It has been suggested that developing embryonic muscles have special characteristic markers or genetic phenotypes that enable appropriate motoneurones to recognize and contact them (Lance-Jones and Landmesser, 1980). But evidence from other studies make this seem unlikely. For instance, it has been shown that even some time after the initial innervation is established the characteristic membrane and contractile properties of all muscle fibres at a comparable
developmental stage are similar (Gordon, Purves and Vrbova, 1977). By surgical manipulation it is easy to innervate embryonic or adult muscle fibres by inappropriate nerves (Langley and Anderson, 1904; Weiss and Hoag, 1946; Summerbell and Stirling, 1981). Finally, muscle fibres have an extraordinary capacity to alter their contractile, ultrastructural and biochemical properties (Biller, Eccles and Eccles, 1960a,b; Ashhurst and Vrbova, 1979; Gordon, Vrbova and Willcock, 1981) and also their phenotype within a wide range in response to changes in activity (Salmons and Vrbova, 1969; Pette, Smith, Staudte and Vrbova, 1973; Pette and Vrbova, 1985). Thus, muscles, rather than recognizing and choosing specific neurones and innervation adjust their properties to the activity they receive from their motoneurones (see Vrbova et al., 1985).

Muscles develop their specific characteristics as postural or flexor muscles as a result of maturation and interaction with their motoneurones during development. This development really begins postnatally.

The most comprehensive study of the development of slow and fast skeletal muscles in the rat comes from Close (1964). He found that initially all muscles are slow. Then after an initial increase in speed slow soleus muscles develop slow contraction speeds. Fast muscles such as the extensor digitorum longus (EDL) however continue to develop increased speeds of contraction and relaxation until adult values are reached.

This change in contraction speeds seen in both slow and fast muscles coincides with the altering pattern of activity imposed on them by their motoneurones. The development of this activity coincides with the development of motility in the animal. Thus, slow soleus
muscles start off being as quiescent as the fast EDL muscles. Gradually, they show an increase in low frequency tonic activity as seen in their EMG until adult values of activity are reached. Fast muscles on the other hand remain quiescent at rest and gradually develop increasingly higher frequency bursts of activity during movement (Navarrete and Vrbová, 1983).

The difference between the dependence of postnatal changes of contractile properties of EDL and Soleus on their innervation can be demonstrated by their response to denervation. If the Soleus muscle was denervated at birth, it continued to increase its speed of contraction until it reached its adult characteristic value for a 20 to 30 day old rat. EDL on the other hand, if denervated, showed no increase in contractile speed (Brown, 1973). This suggested that EDL was dependent on the gradual development and imposition of high frequency activity to bring about the changes in its contractile properties.

Consistent with this was the finding that in the rat, sciatic nerve injury within the first week of life permanently altered the properties (biochemical and electrical) of fast muscles upon reinnervation (Navarrete and Vrbová, 1984; Lowrie et al., 1982; 1984; 1987). In addition if the sciatic nerve was crushed at 5 days of age, fast muscles upon reinnervation recovered only upto 50% of the weight and tension achieved by their contralateral controls although no motoneurone loss was seen (Lowrie et al., 1982; 1984). These reinnervated muscles became more fatigue resistant and in keeping with this they stained darkly for succinic dehydrogenase an enzyme that shows the oxidative capacity of muscles. Normal fast muscles are
highly fatiguable and show darkly stained oxidative fibres interspersed with large pale fibres in a mosaic pattern (Lowrie et al., 1982; Lowrie and Vrbová, 1984; Lowrie et al., 1987).

Consistent with the alteration of their biochemical properties reinnervated fast muscles also showed an increase of up to 2 to 3 times in their electrical activity (EMG) compared to their contralateral controls or to normal animals (Navarrete and Vrbova, 1984). Since a muscle is thought to mirror the activity of its motoneurone (see Vrbova et al., 1985), it was thought that after neonatal nerve crush, the motoneurone too became more excitable (Navarrete and Vrbová, 1984; Vrbová et al., 1985; Navarrete et al., 1986).

All the evidence cited above suggested that the changes seen in both the motoneurone and its muscle as a result of nerve injury occurred because of temporary absence of interaction between the two. Previous studies have shown that both motoneurones (Kashihara et al., 1987) and muscle fibres (Lowrie et al., 1984) did not degenerate immediately after axotomy or nerve crush distal to the soma. Motoneurones remained for up to 2 weeks without a peripheral target after axotomy and only died when they were not allowed to regenerate (Kashihara et al., 1987). Fifty percent of the muscle fibres that were lost from the fast muscles after neonatal nerve injury began to degenerate and die only upon reinnervation (Lowrie et al., 1984). In fact, young denervated muscle fibres were not lost until later (Lowrie and Vrbová, 1984).

These two studies clearly showed the interdependence of motoneurones and muscles on each other and the importance of matching their properties in order that they could both survive as a smoothly functioning unit.
Slow soleus muscles on the other hand, reinnervated after a 5 day crush made an almost complete recovery (Lowrie et al., 1982; 1984; 1987). One reason for its good recovery could be that the soleus did not require the precise matching of properties with its motoneurone (see Brown, 1973; but also recent results of Elmubarak and Ranatunga, 1988) since the pattern of activity imposed on it by its motoneurone did not alter from slow to fast as it did in the case of EDL or TA (Navarrete and Vrbová, 1983).

Another reason for the greater impairment of fast muscles after neonatal nerve injury could be that they became reinnervated later than did the slow muscles so that the period of denervation would have been longer.

The present study was undertaken to establish 2 things: a) the exact period of denervation by following the progress of functional recovery; and b) the changes of contractile properties that took place in fast and slow muscles during the period of denervation, and the reversal of these changes upon reinnervation after sciatic nerve crush at 5 days.
2.2 Methods.

2.2.1. Surgery.

In thirty 5-day old Wistar rats (bred in the animal house in University College) the sciatic nerve was exposed in the middle of the thigh and crushed (9 to 10mm away from the entry of the peroneal nerve into the peroneal muscles) with fine watchmakers forceps. The operation was performed under ether anaesthesia and using sterile precautions. During the crush injury care was taken to preserve the epineurium, to facilitate regeneration of the nerves along their endoneural sheaths. This was done by visual control using a dissecting microscope. The skin was sutured and when the animals recovered from the anaesthetic they were returned to the mother.

2.2.2. Return of Function after nerve injury: Behavioural observations.

Sciatic nerve crush as described above causes the paralysis of the muscles used for movement of the ankle and the toes. The return of function can be assessed non-invasively by scoring the return of the toe-spreading reflex and movement of the ankle.

In this study, return of the toe-spreading reflex and dorsiflexion of the ankle were assessed by a scale devised to score the recovery of function. The animals were observed daily for the return of function in the lower limb. Table 2.1 shows the scale devised to score the return of function. The scale goes from no abduction of the toes or dorsiflexion of the ankle (scored as a minus sign) to when the animal regained the toe-spreading reflex and dorsiflexion of the ankle (scored by a star).

2.2.3. Tension Recordings.

At varying times from 5 to 15 days after nerve crush, (i.e. in animals aged 10 to 20 days old, divided into 3 groups according to the time after nerve crush: Group 1: 7-10; Group 2: 11-12; Group 3: 13-15) the animals were
Table 2.I.
The Table shows the scale devised to assess behavioral recovery after bilateral or unilateral nerve crush. When there was no movement of the ankle or toes, and the lower limbs appeared paralysed, it was thought that reinnervation had not yet begun. A movement through less than 20 degrees was scored with a + sign as the start of reinnervation. Reinnervation was thought to be complete when toes-spreadiong had returned and dorsiflexion of the ankle was similar to that produced by uninjured litter mates. This was scored as a star.
| - | no toe-spreading or dorsiflexion. |
| - | some toe-spreading and dorsiflexion, less than 20 degrees. |
| + | 20 degrees : increased toe-spreading and dorsiflexion |
| ++ | 30 to 40 degrees dorsiflexion and more toe-spreading |
| +++ | More toe-spreading and dorsiflexion |
| ++++ | Increased toe-spreading and dorsiflexion |
| ++++++ | 55 to 65 degrees dorsiflexion, toe-spreading almost back to normal. |
| * | Dorsiflexion and toe-spreading complete. |
prepared for recording of muscle tension. They were anaesthetized with 3.5% chloral hydrate (1ml per 100g body weight). The distal tendons of EDL, TA and Soleus were dissected out, the animal fixed to the table and its tendons attached to strain gauges (Devices Dynamometer UFI of a sensitivity upto 100 grams). The sciatic nerves were dissected in the thighs and sectioned. The distal stump of the cut nerve was placed over bipolar silver electrodes above the original crush injury. Isometric contractions when present, were elicited by stimulating the distal stump of the cut end of the nerve using supramaximal square wave pulses. The length of the muscles was adjusted until a maximal twitch was produced. Single twitch and tetanic tensions were displayed on and photographed from an oscilloscope screen. At the end of each experiment the muscles were dissected out and weighed. The strain gauges were calibrated using gram weights in order to determine muscle forces that had been recorded. The animals were kept at a constant temperature throughout the experiment to minimise variations in muscle tensions.

2.2.4. Muscle histology on completion of physiological measurements.

The soleus and EDL muscles were removed from the operated and control sides of the same animal pinned out in a slightly stretched position on a Sylgaard coated Petri-dish and covered with fixative (4% formaldehyde see O’Brien et al., 1978) for 2 hours. After the muscles were washed, longitudinal frozen sections were cut at 25 μm on a freezing microtome (Pelcool). Every other section was collected to avoid sampling the same area twice. The end-plates and nerve axons were then visualised using a combined cholinesterase and silver stain (Namba, Nakamura and Grob, 1967; modified by O’Brien et al., 1978).

(For detailed protocol refer to Appendix I)
2.3 Results.

2.3.1. Observations of recovery of limb function after nerve crush using behavioural criteria.

After a unilateral sciatic crush at 5 days, young rats were tested for the onset of recovery in the injured leg. This was done by lifting the animals off the ground and observing whether toe-spreading and dorsiflexion took place in the operated leg.

Using the scale shown in Table 2.1 it was observed that toe-spreading and dorsiflexion began at 10 days after the nerve crush and proceeded so that by 13 to 15 days after sciatic nerve crush toe-spreading and dorsiflexion had completely recovered. First signs of recovery of limb function using these behavioural criteria occurred 3 days after the first contractile or EMG response could be elicited by stimulation of the regenerating nerve.

2.3.2. Recovery of muscle function during reinnervation.

Two animals were prepared for tension recordings 6 days after nerve injury and no mechanical or electrical response could be recorded by stimulation of the regenerating motor nerve. At 7 days after the crush, a weak contraction was seen in all three muscles studied when the regenerated sciatic nerve was stimulated. This showed that the regenerating nerve had covered the distance of 10 mm in 7 days, and that the calculated rate of regeneration of the nerve was 1.43 mm per day. At 7 days after the injury reinnervation had only just begun and only a few motor axons had contacted some muscle fibres to produce the small force which was seen when recovery of mechanical function was assessed. On histological examination using the silver cholinesterase stain, it was found that this was indeed the case. Figures 2.1 and 2.2 show photographs of sections of control and
Control (a) and operated EDL muscles prepared for silver cholinesterase are shown here. The muscles are from an animal aged 13 days, and are taken 8 days after nerve crush. The animal fell in Group 1, which consisted of data from animals 7-10 days after sciatic nerve crush at 5 days. It can be seen from Figure 2.1 (b) that in the muscle undergoing reinnervation fine axons are seeking out endplates. The control muscle (a) has the appearance of a normal age-matched EDL muscle. The calibration bar represents 50 um.
operated EDL and Soleus muscles 8 days after nerve crush. These histological findings confirmed that the reinnervating axons had reached both EDL and Soleus muscles and that they had begun to make contact with the muscle fibres. As reinnervation proceeded, more axons were seen to contact muscle fibres.

2.3.3. Recovery of contractile properties of injured muscles.

Single twitch and tetanic responses to stimulation of the regenerating sciatic nerve were seen to be increasing in force as reinnervation proceeded throughout the period of this study which lasted from 7 to 15 days after the initial injury. The mechanical responses were assessed at 3 different time intervals after the nerve injury. In group 1 control and operated muscles were examined 7 to 10 days after nerve crush. In Group 2 muscles were tested 11 to 12 days and in Group 3, 13 to 15 after nerve crush. Examples of tension records from control and operated soleus and EDL muscles in response to single and tetanic stimuli at 20 to 80 Hz are shown in Figures 2.3 and 2.4. The records of Soleus are from an animal at 8 days and those of EDL are from one 15 days after sciatic nerve crush.

The tension developed by reinnervated Soleus, EDL and TA muscles of animals in Group 1 in response to tetanic stimulation at a frequency of 40 Hz was similar to that developed in response to a single shock. This applied to all three muscles studied. The contralateral control muscles whose nerves had been left intact developed much higher tension when stimulated at 40 or 80 Hz than when stimulated by a single shock. The figures show that the operated muscles were much weaker than the control muscle although they were seen to improve as reinnervation proceeds. In addition, neither of the operated muscles were able to maintain tetanic tensions at frequencies
Figure 2.3.
This Figure shows examples of tension records from control (con) and reinnervated (op) Soleus muscles taken from an animal from Group 1, 8 days after sciatic nerve crush in response to single and tetanic shocks at frequencies between 20 and 80 Hz. The reinnervated Soleus was much weaker than its contralateral control as indicated by the vertical calibration. In addition, unlike its contralateral control, (con) the reinnervated muscle (op) was unable to maintain tetanic tensions above 40 Hz (see arrows).
Figure 2.4.
This Figure shows tension responses of control (con) and reinnervated (op) EDL muscles to single and tetanic shocks from 20 to 80 Hz. The records are taken from an animal from Group 3, 14 days after sciatic nerve crush. Once again it is apparent from the vertical calibration bars that the reinnervated muscles were weaker than their contralateral controls and were unable to maintain tetanic tensions at frequencies above 40 Hz (see arrows).
above 40 Hz.

A. Recovery of tension and muscle weight after sciatic nerve crush at 5 days.

Throughout the study, (7 to 15 days after sciatic nerve crush) maximal tetanic tension of the Soleus muscle was obtained by stimulation of its motor nerve at 40 Hz. But, in EDL and TA maximal tetanic tension was obtained in Groups 1 and 2 (7 to 12 days after sciatic nerve crush) by stimulation at 40 Hz and in Group 3 (13 to 15 days after sciatic nerve crush), at 60 to 80 Hz.

Maximal tetanic tension produced by the reinnervated muscles to stimulation of the nerve increased as reinnervation proceeded. The control muscles too produced increased force to stimulation of the control uninjured nerves as the animals were still growing and had not yet attained adult levels. However, the amount of force produced by the muscles undergoing reinnervation was less than that produced by their contralateral control muscles (see Figures 2.3 and 2.4). The maximal tetanic tension produced by the reinnervated muscles was expressed as a percentage of that produced by their contralateral controls and Figure 2.5 summarizes the change in maximum tetanic tension produced by reinnervated muscles throughout the study, 7 to 15 days after sciatic nerve crush. Means ± S.E.M. of maximum tetanic tension expressed as percentages of their control muscles are shown. Both reinnervated soleus and EDL showed some improvement. Soleus increased from 22% to 38% of its contralateral control value and EDL rose from 24% to 32% of its control value. Operated TA showed the least improvement over the course of the 7 to 15 days after nerve crush, increasing from just 12% to 18% of its contralateral control.
Mean ± S.E.M. (N=3 to 6) of maximum tetanic tension of reinnervated muscles expressed as percentage of their contralateral controls are shown in this Figure. Muscles from animals studied 7 to 10 (Group 1), 11 to 12 (Group 2), 13 to 15 (Group 3) days after nerve crush are presented here. Note the dramatic increase in tetanic tension upon reinnervation of the Soleus muscles in Group 3 (clear squares, 22% to 38%) in contrast to the moderate increase in both TA (filled rectangles) and EDL (filled diamonds).
Maximum Tetanic Tension % control

Soleus
EDL
TA

GROUP1  GROUP2  GROUP3
days after crush

83
Mean weights of reinnervated muscles expressed as a percentage of their controls are summarized in Figure 2.6. For the reinnervated soleus muscle, the maximum tetanic tension correlated with its increase in weight. Thus, mean muscle weights of reinnervated soleus when expressed as a percentage of their contralateral controls were 55% at the start of the study and increased to 60% that of their contralateral controls by the end of the study. Although TA and EDL showed an increase in both twitch and tetanic tensions, this appeared to have reached a plateau by the end of the present study. Coupled with this was the finding that the weights of these 2 muscles which at the start of the study were similar to their contralateral controls (Group 1, 7 to 10 days after nerve crush) actually declined as reinnervation proceeded. The relatively low muscle weights of reinnervated TA and EDL, expressed as a percentage of their contralateral controls was mirrored in their actual weights in milligrams. This is summarised in Table 2.II. The Table shows mean weights + S.E.M. of control and operated EDL, TA and Soleus muscles 7 to 10 (Group 1), 11 to 12 (Group 2) or 13 to 15 (Group 3) days after the nerve injury.

Whether the contractile speed alters with the time of reinnervation was assessed next.

**Time to peak twitch contraction.**

As a result of nerve crush the contractile speeds of all three of the muscles were affected. Figure 2.7 shows examples of control and operated EDL (a) TA (b) and Soleus (c) muscles 10 days after nerve crush. The reinnervated muscle in each of the pictures was slower to reach its peak twitch contraction than its contralateral control. Figure 2.8 summarises the results of time to peak contraction obtained
Figure 2.6.
Means of weights of EDL (black bars), TA and Soleus (hatched bars) expressed as a percentage of control are shown in this Figure. Group 1 contained animals which were 12 to 15 days old 7 to 10 days after nerve crush. Groups 2 and 3 contained animals studied 11 to 12 days after nerve crush and 13 to 15 days after nerve crush respectively. As can be seen from the figure, weights of EDL and TA remained between 60% and 80% of contralateral controls although they began to decline as reinnervation proceeded. Soleus however, showed a steady increase in weight as reinnervation proceeded although it seemed most affected on denervation.
muscle weight (% op/con)

EDL  |  TA  |  SOLEUS

GROUP1  |  GROUP2  |  GROUP3

days after crush

0  |  20  |  40  |  60  |  80  |  100

0  |  20  |  40  |  60  |  80  |  100

GROUP1  |  GROUP2  |  GROUP3

EDL  |  TA  |  SOLEUS

85
Table 2.II
This Table shows the mean and standard errors of the mean for the muscle weights of control and reinnervated EDL, TA and Soleus at the various stages of the study. The control muscles were always heavier than the reinnervated muscles. The weights of all three control muscles indicated the growth and development of the animals. The operated muscles too showed an increase in weight but this was not proportional to the growth of the animals. In fact, as reinnervation proceeded, it can be seen that the weights of the reinnervated fast muscles declined when compared with their contralateral controls. The soleus, however, which was most affected upon denervation, gained in weight as the study proceeded.
# Table 2.11.  

<table>
<thead>
<tr>
<th>DAYS AFTER CRUSH</th>
<th>EDL</th>
<th>TA</th>
<th>SOLEUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>9.77±1.75</td>
<td>35.3±7.76</td>
<td>10.32±1.61</td>
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<tr>
<td>(N=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPERATED</td>
<td>7.64±1.88</td>
<td>28.64±6.71</td>
<td>5.11±1.52</td>
</tr>
<tr>
<td>(N=4)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>11-12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>12.88±3.58</td>
<td>47.9±5.71</td>
<td>12.6±1.94</td>
</tr>
<tr>
<td>(N=5)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>OPERATED</td>
<td>11.5±2.58</td>
<td>32.67±4.61</td>
<td>6.71±1.3</td>
</tr>
<tr>
<td>(N=8)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>13-15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>14.21±3.18</td>
<td>60.3±9.66</td>
<td>17.8±3.93</td>
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<tr>
<td>(N=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPERATED</td>
<td>10.14±2.88</td>
<td>38.55±6.96</td>
<td>10.13±1.36</td>
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<td>(N=8)</td>
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Figure 2.7.
This Figure shows examples of single twitches in response to stimulation of the motor nerve of control and operated EDL (A), TA (B) and Soleus (C) muscles. In each case the reinnervated muscle was slower to reach its peak contraction and relax than its contralateral control. These records were taken from an animal from Group 3, 13-15 days after unilateral sciatic nerve crush at 5 days.
Figure 2.8.
This Figure shows the mean ± S.E. of time to peak contraction of control and reinnervated EDL, TA and Soleus muscles after nerve crush at 5 days. The vertical axis shows time in milliseconds. Clear bars are means of control muscles and filled bars those of reinnervated muscles, 7-10 (Group 1, N=6), 11-12 (Group 2, N=7) or 13-15 (Group 3, N=8) days after nerve crush. The differences between the means of operated and control muscles were found to be statistically significant for all three groups for EDL muscles (p<0.01) and for Groups 1 and 2 only for TA. At no time after nerve crush was there a significant difference between the mean time to peak of reinnervated and control Soleus muscles.
time to peak (ms)

EDL

GROUP1  GROUP2  GROUP3

control
operated

TA

GROUP1  GROUP2  GROUP3

control
operated

Soleus

GROUP1  GROUP2  GROUP3

control
operated

days after crush

88
from all three groups. Although the speed of contraction of TA and EDL gradually increased as reinnervation proceeded, these muscles were still contracting more slowly at the end of the study (15 days after nerve crush) than their contralateral controls. The time to peak contraction of the reinnervated Soleus was at no time much different from its control. This may have been due to the fact that control TA and EDL muscles were already faster than the Soleus in all 3 groups. At two months after nerve injury the speed of contraction of reinnervated muscles was not significantly different from their contralateral controls (see Lowrie et al., 1982; 1987).

C. Time to half relaxation.

As can be seen from Figure 2.7, that not only did the operated muscles take longer to reach peak twitch contraction but they also were unable to relax as quickly as their contralateral control muscles. Figure 2.9 summarizes these results. Mean ± S.E.M. of half-relaxation time were calculated for both reinnervated and control muscles 7 to 10, (Group 1) 11 to 12 (Group 2) or 13 to 15 (Group 3) days after nerve crush. In the operated TA and EDL muscles, this characteristic too recovered as reinnervation progressed. Again soleus was closer to its contralateral control value than were either TA or EDL. These properties recover completely 2 months after sciatic nerve crush at five days in rats (see Lowrie et al., 1982; 1987).

D. Twitch/tetanus ratios.

The tension developed by reinnervated EDL and TA muscles of animals in Group 1 in response to tetanic stimulation at a frequency of 40 Hz was similar to that developed in response to a single shock.
Figure 2.9
This Figure shows the means ± S.E. of time to half-relaxation of twitch contraction for control and reinnervated EDL, TA and Soleus muscles after nerve crush at 5 days. The vertical axis shows time in milliseconds and the horizontal axis shows the number of days after nerve crush. Clear bars are means of control muscles and hatched bars are those of reinnervated muscles, 7-10, (Group 1, N=6), 11-12 (Group 2, N=7), and 13-15 (Group 3, N=8) days after nerve crush. The differences between the operated and control means were found to be significant (p<0.01) for EDL and TA in each of the three groups. However, for Soleus there was a significant difference between the mean half relaxation times of operated and control muscles only in animals from Group 1.
This was not the case for the Soleus muscle at the same stage (see Figure 2.3). Moreover, the relationship between the tension developed during twitch and tetanic contraction changed as reinnervation progressed. Thus, it was thought important to measure twitch/tetanus ratios in all three muscles studied.

Twitch/tetanus ratios of the muscles were calculated and these correlated well with their functional recovery. The ratio seems to provide a good indication of the maturity of a muscle. Twitch/tetanus ratios were calculated for all experiments using the following formula taken from Close 1964: \( \frac{P_t}{P_o} \) where \( P_t \) is the maximum twitch and \( P_o \) the maximal tetanic tension produced by a given muscle. The results are summarised in Figure 2.10. Means and standard errors of the values from several operated and control EDL, TA and Soleus muscles are represented here. The Figure shows that twitch/tetanus ratios for operated TA and EDL muscles were higher immediately upon reinnervation (for animals in Group 1) but fell to near contralateral control levels by days 11 or 12 after the injury (animals in Group 3). Interestingly, in none of the groups was the operated Soleus very different from its control, indicating that upon the onset of reinnervation, its recovery was more rapid than either TA or EDL. Other studies have shown that twitch/tetanus ratios for all three muscles return to normal upon completion of reinnervation (and 2 months after the injury) after a similar injury (Lowrie et al., 1984).
Figure 2.10.
Mean ± S.E. twitch/tetanus ratios for control and reinnervated EDL, TA and Soleus muscles are presented here. Clear bars represent means of twitch/tetanus ratios obtained from reinnervated EDL, TA and Soleus after sciatic nerve crush at 5 days. Groups 1 (N=6), 2 (N=7) & 3 (N=8) indicate the time after injury (see Figure 2.9). The y-axis shows the twitch/ tetanus ratio obtained thus: P /P , where P was the maximum twitch t o t tension and P was the maximum tetanic tension produced by the o muscle. The twitch tetanus ratios in all three operated muscles were higher than in the control muscles at the start of the study and gradually returned to towards normal values as reinnervation progressed. These were significantly different from each other (p<0.01) in Groups 1 and 3 for EDL muscles, Groups 1 and 2 for TA and not significantly different for the Soleus muscle in any of the three groups.
twitch-tetanus ratio

EDL

GROUP1  GROUP2  GROUP3

GROUP1  GROUP2  GROUP3

TA

GROUP1  GROUP2  GROUP3

GROUP1  GROUP2  GROUP3

Soleus

GROUP1  GROUP2  GROUP3

days after crush

control
operated

control
operated

control
operated

0.0  0.2  0.4  0.6  0.8

0.0  0.2  0.4  0.6  0.8

0.0  0.2  0.4  0.6  0.8

0.0  0.2  0.4  0.6  0.8
2.4 Discussion.

The results of this study have shown that after sciatic nerve crush at 5 days, the first signs of mechanical and functional recovery occur simultaneously in the three muscles studied. Figures 2.1b (reinnervated EDL) and 2.2b (reinnervated soleus) confirmed this histologically. Thus, there appears to have been no difference in the time of arrival of regenerated axons to either Soleus, TA or EDL muscles. The distance of 10mm from the point of the crush was covered by the axons within 7 days.

Although maximal tetanic tension was seen to increase in all 3 muscles examined as reinnervation proceeded, the soleus muscle showed the greatest improvement increasing from 22% to 38% of its contralateral control. The total increase of mean maximal tetanic tension of soleus muscles from Group 1 (7 to 10 days after crush), to Group 3 (13 to 15 days after crush), was 42% (see Figure 2.5). This was not the case for the fast muscles. In reinnervated EDL muscles after an initial increase in mean maximal tetanic tension upon reinnervation, from 24% (Group 1) to 28% (Group 2) of their contralateral controls it reached 32% of control at the end of the study. Reinnervated TA muscles showed a similar plateauing of the amount of force they could produce in relation to their contralateral controls although the mean force they produced was a significantly smaller percentage and was 12% for Group 1 to 18% and 20% of contralateral controls for Groups 2 and 3 respectively (see Figure 2.5).

The weights of the reinnervated soleus muscles were consistent with the changes in tension produced by them. The weights of fast muscles however seemed remarkably unaffected by the temporary
denervation, (see Figure 2.6) and were similar to their contralateral control muscles. Interestingly, as reinnervation proceeded the weights of the reinnervated TA and EDL increased much less than their controls so that by days 13 to 15 after the crush (Group 3) they weighed only 60% of the control TA and EDL.

Thus, it would appear that although there was no difference in the time of arrival of the regenerating axons to either fast or slow muscles, there was a difference in their rate of recovery. This could be due to the receptivity of the muscle fibres to the ingrowing axons or to the particular types of axons reinnervating them and their special characteristics. So, the soleus muscle which was more affected by denervation (see Figures 2.5 and 2.6), was better able to accept the regenerated axons than were either TA or EDL.

This brings back the idea of matching properties of motoneurones and muscles considered in the Introduction (see section 2.1) to this chapter. Soleus motoneurones which are known to fire at low frequencies (see Navarrete and Vrbova, 1983), do not impose patterns of activity that are incompatible with the arrested development of their temporarily denervated targets. Additionally, it has been shown that young denervated soleus did not change its speed of contraction during the period of denervation whereas an age-matched EDL showed a decrease. Thus, it would appear that immature soleus was better equipped to accept reinnervation and be matched to the properties of its motoneurones than were either EDL or TA. Whether this was because of the characteristic properties of their developing motoneurones or whether it was due to an intrinsic difference between the muscles themselves remains as yet unresolved (see Vrbova et al., 1985 for review). A cross-reinnervation experiment such as performed by Buller
et al.,(1960a,b) on cat muscles could be done to check if it is indeed the motoneurone and its activity that is vitally important for the development of muscle characteristics.

From Figure 2.10 it is clear that twitch/tetanus ratios either did not change, or recovered very rapidly in Soleus whereas the TA and EDL showed considerable changes. The mean twitch /tetanus ratio remained at 0.40 for reinnervated soleus muscles throughout the study whereas, that of reinnervated EDL and TA was 0.70 at the start of the study and fell to near contralateral control levels towards the end of the study. The twitch/tetanus ratio is thought to provide an indication of the ability of a muscle to handle calcium (see Martonosi, 1982). The fact that the reinnervated fast muscles produced twitches comparable to their maximal tetanic tensions could be interpreted as an impairment in their ability to remove the calcium released during excitation-contraction coupling (see Close, 1964).

Consistency of the results of the present study with those of previous ones.

The results of the present study provide an indication as to why there might be a better recovery of the slow Soleus muscle after sciatic nerve crush. They may explain the results of a previous study in which reinnervation of Soleus TA and EDL was followed at later stages after a similar injury. The plateauing of tension recovery of the reinnervated fast muscles seen at the end of this study was shown initially to continue and then the relative tension was shown to actually decrease to 35% to 40% of contralateral control muscles at 3 months after the initial injury (see Figure 2.11 copied from Figure 3 Lowrie and Vrbová, 1984). In the same study, (Lowrie and Vrbová,
**Figure 2.11.**

This Figure is taken from Lowrie and Vrbova (1984) to show the plateauing of recovery of tension in reinnervated fast muscles, TA and EDL that was also seen at the end of the present study. In contrast, reinnervated soleus muscles showed a steady recovery of tension until they produced forces of up to 80% that of their contralateral control muscles. This was in agreement with the results of the present study.
Fig. 3. The recovery of muscle tension following nerve crush. The maximal tetanic tension of each reinnervated muscle was expressed as a percentage of the contralateral control value. Each point represents the mean ± s.e. of the mean of six to ten muscles. The animals were grouped according to age as in Fig. 1. ●, t.a.; ○, e.d.l.; ▲, soleus. Arrow shows time of nerve crush.
the soleus muscle showed a similar increase in tension as it did in the present study. It rose to 80% of its contralateral control value at 3 months after the injury in the previous study (see Figure 2.11 copied from Lowrie and Vrbová, 1984, Figure 3).

Consistent with the results of the present study, Lowrie and Vrbová (1984), found that weights of operated soleus increased upon reinnervation and that those of TA and EDL decreased. Interestingly, it has been found that the soleus muscle, if left denervated degenerated more rapidly than did EDL (Beránek, Hník and Vrbová, 1957; Stewart, 1968; Lowrie and Vrbová, 1984).

In conclusion then, this study showed: a) temporary denervation by sciatic nerve crush at 5 days arrested the development of muscles. This was seen as an initial drop in tension in all muscles accompanied by the arrested development of their contractile properties. The muscle weights of soleus were more affected upon denervation but increased during the course of the study.

b) The main finding of the study was that all muscles were reinnervated at the same time. However, the soleus muscle showed a more rapid recovery of all the parameters studied. This suggested that during early postnatal development, fast muscles required constant interaction with their motoneurones to be able to undergo the developmental changes that make them compatible with their innervating motoneurones.
CHAPTER 3.

Recovery of fast leg muscles, Tibialis Anterior and Extensor digitorum longus after peroneal nerve crush at 5 days at different distances from the muscles.

3.1 Introduction.

During early postnatal life, if nerve-muscle interaction is disrupted, both the muscles and the motoneurones that innervate them become severely impaired. Thus, for example, in rats, injury to immature motoneurones by sciatic nerve crush immediately after birth results in the degeneration and death of 70% of the motoneurones (Romanes, 1946; Bueker et al., 1951; Lowrie et al., 1987). The number of motoneurones that die decreases if the injury is inflicted later after birth so that by day 5 in rats no motoneurone loss is seen as a result of sciatic nerve crush (Romanes, 1946; Lowrie et al., 1982; 1984; 1987).

This has been demonstrated in several other species too. Hess,(1956; 1957) using guinea pigs has shown a progressive decrease with age in the vulnerability to injury of spinal neurones both pre- and postnatally. La Velle and La Velle (1959), and La Velle (1964) have demonstrated that injuries to the facial nerve inflicted in 20 day old hamsters resulted in the loss of only a few neurones whereas in 7 to 15 day old animals, many more were lost.

The effects of such injuries as described above have been shown to result in degenerative changes in muscles previously innervated by the injured motoneurones. Muscles disconnected from their motoneurones early in embryonic development grow for a short while and then disintegrate (Eastlick and Wortham, 1947). If at this time innervation
is left intact but transmission from nerve to muscle is prevented, degenerative changes are seen in the muscle fibres (Srihari and Vrbová, 1978). Permanent denervation later in embryonic development seems to have a less severe effect on muscle fibres, so that, although some degenerate others remain and continue to grow slowly (Stewart, 1968).

In mammals, the severity of the effects of temporary denervation on muscle in early postnatal life also depends on the age at which the injury is inflicted. Several authors have found that the weights and muscle fibre numbers of EDL, TA and soleus never reached contralateral control levels after sciatic nerve crush at birth (Zelena and Hnik, 1963; McArdle and Sansone, 1977). In contrast, if the sciatic nerve was crushed when the animal was 14 to 35 days old, the reinnervated muscles recovered to the control levels (McArdle and Sansone, 1977; Lowrie et al., 1982).

Zelena and Hnik (1963), have shown that after sciatic nerve crush at birth, the soleus muscle had a smaller number of muscle fibres though their mean diameter was significantly larger than normal. The number of motor nerve fibres was reduced to 50% that of contralateral controls 6 to 7 months after the operation. Mean nerve fibre diameters were also reduced by 25%. They also found that motor units were smaller despite the fact that reinnervated muscle fibres were larger than those of normal contralateral control muscles. Thus, the loss of muscle fibres could be attributed to the loss of motoneurones supplying them.

However, if a similar injury was inflicted in adult rabbit or rat muscles and motoneurones, they recovered completely. No muscle fibres or motoneurone loss was seen (Gutmann et al., 1944; Beránek et al.,
Although sciatic nerve crush in 5 day old rats did not result in motoneurone death, the functional recovery of the fast muscles TA and EDL remained poor (Lowrie et al., 1982; 1984). This was so because muscle fibres were lost. Weights and tensions of reinnervated TA and EDL remained at 50% of those of their contralateral control muscles.

Surprisingly, reinnervated slow soleus muscles showed much less permanent impairment (Lowrie et al., 1982; 1984; 1987). Thus, motoneurone loss could not explain the selective impairment seen in TA and EDL upon reinnervation after sciatic nerve crush at 5 days.

Since fast muscles such as TA and EDL were known to have their full complement of fibres at birth (Zelená, 1962; Ontell, 1979), the loss of fibres seen after sciatic nerve injury at 5 days could not be attributed to an inhibition of the formation of new muscle fibres. Neither could the loss of muscle fibres be due to the temporary denervation, for all the fibres were seen to be present at the time the nerve returned to reinnervate the muscle. Muscle fibres were seen to be lost only upon reinnervation by regenerated axons (Chapter 2 this thesis, Lowrie and Vrbová, 1984).

In addition, reinnervated fast muscles after unilateral sciatic nerve crush inflicted neonatally became very fatigue resistant upon repeated stimulation, a characteristic very different from their contralateral control muscles. Normal fast skeletal muscles of adult rats are known to fatigue on repeated stimulation. (Lowrie et al., 1982).

Consistent with the increased fatigue resistance was the appearance of the muscle fibres when stained with succinic
dehydrogenase. This enzyme reveals the oxidative capacity of muscles. Thus, those fibres with more oxidative activity stain darkly and those with low activity stain palely. That the increased fatigue resistance of fast muscles reinnervated after neonatal sciatic nerve crush was not due only to the injury per se but also depended on its timing was confirmed by a similar injury inflicted on adult muscles (Lowrie et al., 1982).

The explanation given by Lowrie et al., (1982) for the slight increase in fatigue resistance of fast muscle fibres after sciatic crush in adulthood was that axons originally supplying oxidative, fatigue resistant muscle fibres occupied on reinnervation a larger territory than before. Or, it was possible that motoneurones with reinnervating axons became more excitable after the injury increasing thereby the overall activity of the muscle.

After unilateral nerve injury in early postnatal life however, the effects were more dramatic. The reinnervated TA and EDL muscles stained uniformly darkly after unilateral nerve crush when their oxidative capacity was tested using succinic dehydrogenase (Lowrie et al., 1982) and the normal mosaic pattern of the contralateral control muscles was not seen at all. Thus, not only were reinnervated muscles more fatigue resistant they were also more active in terms of their oxidative activity.

Based on this and previous evidence on the development of muscle activity patterns (Navarrete and Vrbová, 1980; 1983; 1984), Vrbová et al., (1985) suggested that when the nerve was crushed at 5 or 6 days, the muscle was isolated from all nervous influence and muscle differentiation was retarded. The motoneurones continued to differentiate and developed central connections despite temporary
disconnection from their targets. Upon reinnervation immature fast muscle fibres that had not already experienced the small amounts of low frequency activity which would be normal for animals of that age to gradually change their properties, may not have been able to withstand the now larger amounts of high frequency activity imposed upon them by the "faster" older motoneurones.

In contrast, soleus muscle fibres may have continued to differentiate despite lack of innervation, so that upon reinnervation they have acquired contractile properties that match the properties of their motoneurones. Since soleus muscle fibres were normally activated by motoneurones that fired at low frequencies which matched the slow time course of contraction of their target fibres, the risk of mismatch would not be as great as that between young denervated fast muscles and their motoneurones.

Thus, this mismatch between young fast muscles and their motoneurones which occurred because of temporary denervation led to their permanent impairment. Vrbová et al., (1985) have concluded their argument by saying that during normal development the contact between nerve and muscle was of greater importance for fast muscles, in which the nerve induced a greater change in their characteristics during this critical time in development (see also Chapter 2 of this thesis). The effects of shortening the period of disruption of nerve muscle interaction.

From 1940-1950 Gutmann and his colleagues investigated the effects of various kinds of nerve lesions on recovery of adult leg muscles of the rabbit. They found that recovery of muscle function after nerve crush occurred earlier when the nerve lesion was made close to
the muscle. However, the rate of advance of the regenerating nerve remained unaltered no matter whether the lesion was made close to or far away from the muscles. As discussed above, recovery of muscle function after nerve lesions made in adult animals was known to be complete (Gutmann et al., 1944; La Velle and La Velle, 1959; Hess, 1956; 1957) upon reinnervation.

Some workers have reported that temporary disruption of nerve-muscle interaction during early postnatal life did not result in the profound impairment of either, if the lesion was made close to the muscle (Brown et al., 1976). However, these authors were studying the slow soleus muscle which was known to recover better after such an insult (see Lowrie et al., 1982; 1984; Vrbová et al., 1985). Their results suggested that permanent impairment of muscle function could be averted if the duration of disruption between the immature nerve and muscle was kept very short.

The experiments described in this Chapter test these possibilities. The results described are from those experiments designed to study the effects of varying the length of time of disruption of interaction between fast muscles and their motoneurones they innervate during early postnatal development.
3.2 METHODS.

3.2.1. Surgery.

In all experiments surgery was performed on 5 day-old Wistar rats (bred by the animal house), using ether anaesthesia and sterile precautions. The animals were divided into 3 groups (A, B and C), and accordingly, 3 different types of operations were performed on them. Table 3.I summarizes the 3 types of operation and depicts the 3 experimental groups used in this study.

In group A, in 5 day old wistar rats (N=16) the Peroneal (P) nerve was exposed in the middle of the right thigh and crushed with fine watchmakers forceps 9 mm away from the muscles tibialis anterior (TA) and extensor digitorum longus (EDL). The epineurium was preserved, thus facilitating regeneration of the nerves along their endoneural sheaths. The wound was then closed. A similar operation was carried out on the left side but here, the Peroneal (P) nerve was exposed just before it entered TA and EDL muscles and crushed 3 mm proximal to the muscles in the manner described above. The wound was then sutured and the animals allowed to recover from anaesthesia before being returned to their mother.

In group B, 5 day old wistar rats (N=6) were anaesthetised with ether and under sterile conditions as before, the P nerve was exposed in the right thigh of the animal. It was crushed as described above with fine watchmakers forceps, the wound sutured and the animals allowed to recover from anaesthesia and returned to their mother. No operation was performed on the left leg the muscles of which served as a normal control within each animal.

5 day old Wistar rats were anaesthetized with ether and their sciatic nerves were crushed in the right thigh with fine watchmakers
Table 3.1.
The Table shows the operations performed on each of the three groups. In group A, the Peroneal (P) nerve was crushed on the left side 3 mm away from the TA and EDL muscles. In the same animal, the P nerve was crushed on the right side 9 mm away from TA and EDL. The animals in Group A were referred to as having had bilateral nerve injury at 5 days old. In Group B, the P nerve was crushed 9 mm away from TA and EDL on the right side. The left side was left intact and served as a normal control. In Group C the sciatic nerve was crushed 9 mm away from TA and EDL on the right side. The left side was left intact as in group B for the same purpose. Group C served as a control experimental group for Groups A and B in which animals had had only the P nerve crushed and not the whole sciatic.
Table 3.1: Summary of types of experiment.

<table>
<thead>
<tr>
<th>Type of Injury</th>
<th>Left Side.</th>
<th>Right Side.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilateral Crush</td>
<td>P nerve crush 3 mm from TA and EDL</td>
<td>P nerve crush 9 mm from TA and EDL</td>
</tr>
<tr>
<td>(group A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilateral Crush</td>
<td>No nerve crush</td>
<td>P nerve crush 9 mm from TA and EDL</td>
</tr>
<tr>
<td>(group B)</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Unilateral Crush</td>
<td>No nerve crush</td>
<td>Sciatic nerve crush</td>
</tr>
<tr>
<td>(group C)</td>
<td>Control</td>
<td>9 mm from TA and EDL</td>
</tr>
</tbody>
</table>
forceps as described before (Group C, N=6). The wound was closed and the animals allowed to recover and return to their mother. Once again, the left leg was left intact and served as a normal control within each animal. Groups B and C are referred to as unilateral crushes and they served as controls to group A which were referred to as bilateral crushes.

3.2.2. Return of function after nerve injury: Behavioural Observations.

With a nerve crush such as described above sustained by animals in groups A, B and C, the muscles used for dorsiflexion of the ankle and toe-spreading become denervated. Thus, until the muscles are reinnervated by the regenerating, injured nerves, the animals remain unable to perform dorsiflexion of the ankle or to spread their toes and have paralysed legs. To assess whether reinnervation is proceeding, observations of the return of these 2 reflexes, toe-spreading and dorsiflexion of the ankle are useful non-invasive means of scoring functional recovery after nerve injury.

Recovery of function of TA and EDL, which were the muscles of interest in this study, was assessed by the return of dorsiflexion of the ankle and the toe-spreading reflex. Animals in all groups (A, B and C) were observed daily after nerve crush until such time as toe-spreading and dorsiflexion of the ankle returned. Table 3.II shows the scale devised to score the return of the function. The scale goes from no abduction of the toes or dorsiflexion of the ankle (scored as a minus sign) to when the animal regained its ability to dorsiflex the ankle and lift the foot in addition to spreading of its toes (scored by a star).
Table 3.II.
The Table shows the scale devised to assess behavioral recovery after bilateral or unilateral nerve crush. When there was no movement of the ankle or toes, and the lower limbs appeared paralysed, it was thought that reinnervation had not yet begun. A movement through less than 20 degrees was scored with a + sign as the start of reinnervation. Reinnervation was thought to be complete when toe-spread had returned and dorsiflexion of the ankle was similar to that produced by uninjured litter mates. This was scored as a star.
Table 3.II.

- no toe-spreading or dorsiflexion.
+ some toe-spreading and dorsiflexion, less than 20 degrees.
+ 20 degrees: increased toe-spreading and dorsiflexion
++ 30 to 40 degrees dorsiflexion and more toe-spreading
++ More toe-spreading and dorsiflexion
+++ Increased toe-spreading and dorsiflexion
++++ 55 to 65 degrees dorsiflexion, toe-spreading almost back to normal.
* Dorsiflexion and toe-spreading complete.
Normally, during dorsiflexion of the ankle, the angle between the foot and the front of the leg is between 70 to 80 degrees. Four bold plus signs (+) denoted that dorsiflexion and abduction were nearly complete. The onset of dorsiflexion was assessed as being an angle of movement of less than 20 degrees and was scored as a single plus sign (+).

3.2.3 Isometric tension recordings in vivo.

At a minimum of 6 weeks after the initial injury, animals from groups A, B and C were anaesthetized with 4.5% chloral hydrate injected intraperitoneally. The initial dose was 1ml per 100g body weight and the level of anaesthesia was checked periodically by assessing pupillary and other reflexes. A further 20% of the original dose was given as required during the course of the experiment. All experiments were carried out at room temperature (20 degrees centigrade). The tendons of the EDL and TA muscles were exposed by a small incision at the level of the extensor retinaculum, the animals fixed down to the cork table at the knee and ankle joints with pins and the tendons dissected free and attached to a strain gauge (Dynamometer UFI) appropriate to the tension developed by the muscles (100 to 500g). The muscles were kept close to body temperature by minimizing their exposure and the animals were kept warm by the heat of a lamp. The exposed parts of the tendons were kept moist with 0.9% sodium chloride solution throughout the experiment. Each of the sciatic nerves were dissected out at their entry into the thigh and cut. The distal stump of the nerve was placed over bipolar silver electrodes above the original point of the crush.

Isometric contractions were elicited from the muscles by stimulating the distal stump of the cut end of the nerve using supramaximal square wave pulses of 50 us duration. The length of
the muscles was adjusted until a maximal twitch was produced. Single
twitch and tetanic (40 to 100 Hz) contractions were amplified and
displayed on an oscilloscope (Tektronix R5113) and photographed for
calculation of tension. Means and standard errors of these (and
other parameters) were calculated for all groups and parametric
(paired and unpaired t-tests) and non-parametric (Kruskall-Wallis
one way analysis of variance by ranks) tests were carried out to
assess the statistical significance of the results. At the end of
all experiments muscles were dissected out and weighed.

3.2.4. Calculation of Fatigue Index.

At the end of some experiments, the EDL muscles were stimulated
repeatedly for 250ms at 40 Hz every second and these contractions
were displayed on a Devices M 8 pen recorder. The decrease in
tension after 3 minutes of such stimulation was measured. A fatigue
index was then calculated as follows:

\[
F.I. = \frac{\text{Initial tension} - \text{tension after 3 minutes}}{\text{Initial tension}}
\]

3.2.5. Muscle Histology.

After some experiments, following the physiological study, the
EDL muscles were removed, weighed and prepared for histology as
follows: Corresponding muscles from each animal were mounted side
by side against a pin embedded in a cork block, at the same length,
and then quickly frozen in melting isopentane, cooled with liquid
nitrogen. Each pair of muscles were subsequently processed as a
single block of tissue. Transverse sections from the middle third of
each muscle pair were cut at a thickness of 10um using a cryostat at
temperatures of between -20 and -25 degrees centigrade. The sections
were mounted on glass slides air-dried and reacted for succinic
dehydrogenase, an enzyme which indicates the oxidative activity of
muscles (for detailed protocol refer to Appendix II).
3.3 Results.

3.3.1. Observations of return of function assessed behaviourally: Return of toe-spread and dorsiflexion of the ankle.

Group A (After Bilateral Nerve Injury).

Onset and completion of functional recovery was always seen earlier on the left side where the nerve crush was 3mm away from TA and EDL (see Table 3.1) than on the right side where the nerve crush was 9mm away from the muscles. Table 3.111 shows means and standard errors for onset and completion of recovery assessed in this fashion for 18 animals which had had bilateral nerve crush at 5 days. Mean onset of recovery after nerve crush 3 mm away from muscles (left side) was between 5 and 6 days, while that for nerves crushed 9 mm away (right side) was between 9 and 10 days. Completion of recovery was between 12 and 13 days on the left side where the P nerve had been crushed 3 mm away from the muscles. It was between 16 and 17 days on the right side where the P nerve had been crushed 9 mm away from the muscles. These results were significantly different from each other at the 0.01 level when a paired t-test was used. Thus, muscles whose nerves had been crushed 9 mm away from them remained denervated for 4 days longer than did those with their nerves crushed 3 mm away.

From the above finding it would appear that axons of young motoneurones covered a distance of 6mm in 4 days. The rate of growth was therefore 1.5 mm per day.
Table 3.III.
The Table summarises the observations of recovery of toe-spreading and dorsiflexion of the ankle in days after P nerve crush close to (3 mm) or far from (9 mm) TA and EDL muscles. The number of animals observed was 18 and the table shows the mean of the recovery of their muscles. A paired t-test showed that there was a significant difference (p<0.01) in the recovery of the reflexes between the left (nerve crush 3 mm from the muscles) and right (nerve crush 9 mm from the muscles) sides of these animals.
Table 3.III.

<table>
<thead>
<tr>
<th>Type of injury</th>
<th>Onset of recovery in days</th>
<th>Completion of recovery in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>P crush 3 mm</td>
<td>5.2±0.13</td>
<td>12.4±0.21</td>
</tr>
<tr>
<td>away from the muscles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P crush 9 mm</td>
<td>9.2±0.29</td>
<td>16.5±0.44</td>
</tr>
<tr>
<td>away from the muscles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Groups B and C (After unilateral nerve crush).

After unilateral crush the behavioural recovery of function followed a similar time course to that of animals with bilateral nerve injury where the nerve had been crushed 9 mm away from the muscles.

3.3.2. Recovery of tension and weight of TA and EDL muscles reinnervated after bilateral P nerve crush (Group A).

A. In this section the recovery of muscles reinnervated after P nerve crush 3 mm away from the muscles on the left side and 9 mm away from the muscles on the right side in the same animal will be directly compared. The degree of final recovery after such a bilateral crush was assessed by the amount of tension developed by reinnervated muscles. Their weights were also compared. This assessment was carried out after reinnervation was complete, 6 weeks to 6 months after the initial injury. The animals were anaesthetised and tension developed by reinnervated TA and EDL muscles was recorded as described in the Methods section of this chapter.

Figure 3.1 shows an example of single twitch and maximal tetanic contractions developed from an animal which had had its P nerves crushed 3 mm away on the left side and 9 mm away from the muscles on the right side. (A) shows responses of the EDL muscle on the left side to stimulation of its nerve by a single shock and at a frequency of 80 Hz 3 months after the initial injury. (B) shows the response of the contralateral EDL from the right side in the same animal to a similar pattern of stimulation of its nerve. As can be seen from the Figure, in this animal, the reinnervated EDL on the left side (A) which had had its nerve crushed only 3 mm away from it was stronger than the reinnervated EDL from the right side which...
Figure 3.1.

Tetanic contractions elicited by stimulation of the motor nerve to EDL muscle are shown in this Figure. In (A) a single twitch and tetanic contraction from the left EDL muscle reinnervated after P nerve crush 3 mm away from it and in (B) the response to similar stimulation of the right EDL muscle which had had a P nerve crush 9 mm away from it are shown. These traces are taken from the same animal. The horizontal bars represent time and the vertical bars represent tension in grams. It is clear from the responses shown here that the muscle which was left denervated for a shorter period of time (P nerve crush 3 mm away) was stronger than its contralateral counterpart.
had had its nerve crushed 9 mm away from it.

Figure 3.2 A summarizes the results obtained from several such experiments. Maximal tetanic tension is shown on the ordinate. Filled bars show means and standard errors for TA and EDL muscles which had their P nerves crushed 3 mm away from them and the hatched bars show means and standard errors taken for muscles which had a P nerve crush 9 mm away. The number of muscles sampled was 12. These results show that TA and EDL were significantly stronger (p<0.001 paired t-test) on the left side where the crush had been only 3 mm away from the muscles than on the right side where the crush had been 9 mm away.

These results showed that the amount of time that a young muscle was left denervated influenced the recovery of its strength.

The recovery of muscle weights was assessed next. After each experiment TA and EDL muscles were removed and weighed to compare the effects of crush close to (3 mm away) and far from (9 mm away) the muscles. The weights of TA and EDL followed exactly the same pattern of recovery as did their strength and this is summarized in Figure 3.2 B. Means and their standard errors are shown for weights of TA and EDL muscles after P crushes either 3 mm away (clear bars) or 9 mm away (hatched bars) from them in 16 animals. The ordinate on the right hand side shows muscle weights in milligrams. Muscles on the left side of the animal with the nerve crush close to them, were heavier than those that had had their nerves crushed 9 mm away from them. Thus, the increased weight of muscles with a neonatal nerve crush close to them seemed directly related to the amount of time that they were left denervated.
Figure 3.2.A & B.

These Figures summarise the data of maximal tetanic tension (A) and muscle weights (B) of TA and EDL after P nerve crush 3 and 9 mm away from them. The vertical axes show mean maximal tetanic tension in grams (N=12) and mean muscle weights in milligrams (N=16). The unfilled bars represent mean values from reinnervated muscles after P nerve crush 3 mm away. The filled bars are mean values from reinnervated muscles after P nerve crush 9 mm away.
muscle weight in milligrams

muscles

TA

EDL

3mm away

9mm away
To assess directly the effects of the distance of the injury on the recovery of the muscles, ratios of maximal tetanic tensions and weights of TA and EDL muscles were calculated. Means and standard errors for each muscle were then calculated for tension (N=12) and weight (N=16). The mean ratios of maximal tetanic tension produced by TA and EDL after nerve crush 3 mm away were 1.38 ± 0.08 (S.E.M.) and 1.45 ± 0.09 (S.E.M.) respectively of that produced by the contralateral muscles which had sustained P nerve crush 9 mm away from them.

The mean ratios for muscle weights of TA and EDL after nerve crush 3 mm away from them were 1.2 ± 0.04 (S.E.M.) and 1.31 ± 0.06 (S.E.M.) respectively of those of the contralateral TA and EDL.

3.3.3. Recovery of time to peak contraction and time to half relaxation of reinnervated TA and EDL after bilateral P nerve crush.

Effects of nerve crush 3 mm away or 9 mm away from the muscles were also tested on other physiological properties of reinnervated muscles such as time to peak contraction and time to half-relaxation and were then compared. These were not found to be significantly different from each other and were within the range of values of normal adult TA and EDL muscles. Values for normal uninjured TA and EDL muscles are known to be between 19 ms and 22 ms for both time to peak and time to half-relaxation (see Lowrie et al., 1982; 1984; 1987). Table 3.IV shows means from 11 animals for TA and EDL after nerve crush close to (3mm) or far from (9mm) the muscles. (A) shows means of time to peak and (B) shows time to half-relaxation. This result shows that these parameters of the muscles were not affected after neonatal nerve injury.
Table 3.IV.A. Time to peak Contraction.
Means ± S.E.M. of time to peak contraction in milliseconds for reinnervated TA and EDL after bilateral P crush from 11 animals are shown here. These were not statistically different from each other. Nor were they different from normal adult values which were between 20 and 23 milliseconds for TA and EDL (see table II Lowrie et al., 1987).

Table 3.IV.B. Time to half-relaxation.
Means ± S.E.M. of time to half-relaxation in milliseconds for reinnervated TA and EDL are shown here for 11 animals which had had P nerve crush 3 mm away from the muscles in one leg and 9 mm away in the other leg. These were not found to be statistically significant from each other or from those of normal adult muscles which were between 18 and 20 milliseconds (see table III Lowrie et al., 1987).
### Table 3.4.

<table>
<thead>
<tr>
<th></th>
<th>Time to peak contraction (ms)</th>
<th>Time to half-relaxation (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muscle</strong></td>
<td>P crush 3 mm away</td>
<td>P crush 9 mm away</td>
</tr>
<tr>
<td>TA</td>
<td>22.45 ± 0.98</td>
<td>23.09 ± 0.86</td>
</tr>
<tr>
<td>EDL</td>
<td>21.18 ± 0.89</td>
<td>22.36 ± 1.23</td>
</tr>
</tbody>
</table>

### B. Time to half-relaxation (ms).

<table>
<thead>
<tr>
<th></th>
<th>P crush 3mm away</th>
<th>P crush 9 mm away</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>20.54 ± 0.8</td>
<td>21.72 ± 0.78</td>
</tr>
<tr>
<td>EDL</td>
<td>21.36 ± 0.82</td>
<td>21.45 ± 0.82</td>
</tr>
</tbody>
</table>
3.3.4. Recovery of twitch/tetanus ratios of reinnervated TA and EDL after bilateral P nerve crush.

Twitch /tetanus ratios were calculated for TA and EDL after P nerve crush close to or far away from them. The ratio was calculated as follows: \( \frac{P_t}{P_o} \) where \( P_t \) was the maximum isometric twitch tension and \( P_o \) was the maximum isometric tetanic tension produced by a given muscle (see Close, 1964). Means of twitch /tetanus ratios for all 4 reinnervated muscles for 10 animals were calculated and were as follows:

Nerve crush 3mm from | Nerve crush 9 mm from
---|---
TA=0.22\(\pm\)0.015 | EDL=0.21\(\pm\)0.01
TA=0.19\(\pm\)0.02 | EDL=0.19\(\pm\)0.01

These values were not significantly different from each other and were consistent with those of normal adult fast muscles (see Close, 1964).

3.3.5. Comparisons of tensions and weights produced by reinnervated TA and EDL muscles from group A with estimates of those produced by normal uninjured TA and EDL muscles (from normal age and weight matched control animals.)

In animals with bilateral P crush such as those described in group A, there was no normal contralateral control side to compare the tensions and weights developed by reinnervated TA and EDL muscles after a nerve crush 3mm or 9mm away from them. In order to make such comparisons, tensions and weights of normal uninjured TA and EDL were obtained from several control animals and a norm was set up whereby the tetanic tension or weight of a given muscle was plotted as a function of its body weight. This showed a positive correlation so that for a given body weight it was possible to estimate the amount of
tension a muscle produced and how much it weighed. Additionally, the amount of tetanic tension produced by a muscle of a given weight could also be estimated (see Figures 3.3 and 3.4). Thus, for animals with bilateral P nerve crush of a given body weight, the normal maximal tetanic tension and muscle weights of TA and EDL could be estimated using values calculated from Figures 3.3 and 3.4. The maximal tetanic tension and muscle weight of the reinnervated muscles after bilateral P crush were then compared to the estimated normal values and expressed as percentages of those normal values. This was done for each experimental animal in group A and means and standard errors calculated for the percentage of estimated normal tension and weight for each muscle. This is shown in Figure 3.5.

When expressed in relation to normal muscles recovery of tension (3.5 A) and weight (3.5 B) was always better on the left side where the nerve was crushed 3mm away from the muscles (filled bars) than on the right side where the nerve was crushed 9mm away from the muscles (hatched bars). Figure 3.5 also shows that despite the shorter duration of disruption between nerve-muscle interaction after nerve crush close to the muscles (3 mm away), the tension and weight of reinnervated TA and EDL was between 45% and 65% that of normal muscles. In fact, the difference between the recovery of the muscles of the 2 reinnervated sides although statistically significant, was only between 10% and 15%. It was also clear that TA recovered less well than did EDL.

The recovery of TA and EDL could have been affected by the bilateral nature of the injury. To test for this possibility, experiments were done in which a unilateral P crush was inflicted on a group of animals 9mm away from the muscles.
Figures 3.3 and 3.4.

These two Figures show the correlation between body weight and muscle weight (a), body weight and maximal tetanic tension (b) seen in normal adult animals (N=10) for TA (Figure 3.3) and EDL muscles (Figure 3.4). In addition (c) of Figures 3.3 and 3.4 is the correlation between the muscle weight and the maximal tetanic tension of normal adult TA and EDL muscles. The values were taken from 10 normal animals and were plotted as either maximum tetanic tension or muscle weight as a function of the body weight of each animal (a and b) or as maximum tetanic tension as a function of muscle weight of each animal (c). To test the correlation between each of the above parameters for each muscle regression lines were calculated. The positive correlations between these characteristics (such as the body weight and muscle weight) and the function of muscles (expressed as the tension they can produce) were used as a norm for the estimation of recovery seen in animals which had had bilateral nerve crushes in the present study.
3.4

(a) Muscle weight (mg)

\[ y = -12.5 + 1.975x \]

(b) max Tet (g)

\[ y = 21.35 + 2.98x \]

(c) max Tet (g)

\[ y = 37.86 + 1.49x \]
Figure 3.5.A.
Maximal tetanic tension of reinnervated TA and EDL expressed in this Figure as percentages of estimated normal weight matched control muscles. The hatched bars are means of tensions from reinnervated muscles after P crush 9 mm away from them. Black bars represent means from the same animals of tensions of reinnervated muscles after P crush 3 mm away from them. These values were significantly different from the estimated normal values (p<0.01) for both muscles.
maximum tetanic tension % est. normal

3mm away
9mm away

muscles
Figure 3.5.B.
Muscle weights of reinnervated TA and EDL expressed in this figure as percentages of estimated normal weight matched control muscles. The hatched bars are means of tensions from reinnervated muscles after P crush 9 mm away from them. Black bars represent means from the same animals of weights of reinnervated muscles after P crush 3 mm away from them. These values were significantly different from estimates of normal muscle weight for both TA and EDL muscles.
3.3.6 Comparisons of tensions and weights of reinnervated TA and EDL muscles after bilateral or unilateral P crush 9 mm away from the muscles.

Table 3.V shows a comparison between tensions and weights of reinnervated TA and EDL muscles after bilateral or unilateral nerve crush 9 mm away from the muscles. The mean tensions and weights are expressed as percentages of normal TA and EDL muscles and were not significantly different from each other using the Students' t-test. This showed that the bilateral nature of the injury did not have a significantly different effect on the recovery of tension and weight of TA and EDL when the nerve was crushed 9 mm away from them when compared to recovery of reinnervated TA and EDL after unilateral P crush 9 mm away from them.

3.3.7 Comparisons of tensions and weights of reinnervated TA and EDL after bilateral P crush, (Group A) unilateral P crush (Group B) and unilateral sciatic crush (Group C) inflicted 9 mm away from the muscles.

In some experiments, the whole sciatic nerve was crushed 9 mm away from the muscles (the same distance as the P was crushed either in the unilateral crush experiments (Group B) or the bilateral crush experiments (Group A)). This was done in order to see if the effects of unilateral or bilateral P crush 9 mm away from the muscles affected their recovery. Figures 3.6 and 3.7 show the comparisons of mean maximal tetanic tension and muscle weights of reinnervated TA and EDL expressed as percentages of normal controls after P and sciatic nerve crush 9 mm away from the muscles. As can be seen from Figure 3.6, it would appear that maximal tetanic tension
Table 3.V.

Comparison of mean maximal tetanic tensions and muscle weights of reinnervated TA and EDL after bilateral P nerve crush 9 mm away from the muscles with those after unilateral P nerve crush at the same distance away from the muscles. This was done in order to compare whether recovery of tension and weight of reinnervated muscles was affected by the bilateral nature of the injury. The mean tensions are expressed as percentages of estimates of normal weight matched controls (for the bilateral crush group) or those of contralateral control muscles (for unilateral P crush group).
<table>
<thead>
<tr>
<th>Muscle</th>
<th>%\text{normal Tension}</th>
<th>%\text{Muscle Weight}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilateral TA</td>
<td>36 ± 3.0 (N=12)</td>
<td>49 ± 3.2 (N=16)</td>
</tr>
<tr>
<td>P crush EDL</td>
<td>45 ± 3.1 (N=12)</td>
<td>49 ± 3.1 (N=16)</td>
</tr>
<tr>
<td>Unilateral TA</td>
<td>42.5 ± 2.7 (N=6)</td>
<td>45 ± 2.0 (N=6)</td>
</tr>
<tr>
<td>P crush EDL</td>
<td>48 ± 3.0 (N=6)</td>
<td>46 ± 2.0 (N=6)</td>
</tr>
</tbody>
</table>
Figures 3.6 and 3.7.

Comparisons of mean maximal tetanic tension (Figure 3.6) and muscle weights (Figure 3.7) of reinnervated TA and EDL expressed as percentages of normal controls after bilateral P crush (bl) unilateral P crush (ulp) and unilateral sciatic crush (usc) 9 mm away from them. Although the recovery of tension of reinnervated TA after bilateral P nerve crush appeared to be lower than that of either unilateral P crush or sciatic crush, this was not statistically significant. EDL was similarly affected after bilateral or unilateral nerve crush.
3.7
produced by reinnervated TA was slightly lower after bilateral P nerve crush than it was after either unilateral P nerve crush or unilateral sciatic nerve crush but this small difference was not statistically significant. As can be seen from both Figures 3.6 and 3.7, EDL seemed to be similarly affected after bilateral or unilateral P crush or unilateral sciatic nerve crush. Thus, provided the nerve crush was inflicted at the same distance from the muscles the effects were similar, no matter whether the sciatic or P nerves were injured.

3.3.8. Fatiguability of reinnervated EDL muscles after bilateral P nerve crush 3 mm away or 9 mm away or after unilateral P nerve crush 9 mm away.

In some experiments, the EDL muscles were stimulated repeatedly for 250 msec at 40 Hz every second as described in the Methods section of this chapter. Figure 3.8 (A) and (B) shows examples of such experiments. The recordings of tension were from EDL muscles taken from two animals, one with a bilateral P nerve crush (A) and the other from an animal with a unilateral P nerve crush 9 mm away from the muscles. The fatigue index was then calculated as described and means and standard errors were calculated for reinnervated muscles in Groups A and B.

The reinnervated EDL muscles were more fatiguable the closer the nerve crush was to the muscle. Thus, after P nerve crush 3 mm away from the EDL muscles the mean fatigue index was 0.55 and was significantly different (p<0.01 using a paired t-test) from that of the contralateral EDL muscle which had had a P nerve crush 9 mm away from it. The mean fatigue index for the latter was 0.43.

The fatigue indices of control and reinnervated EDL muscles after
Figure 3.8.
A. An example of a fatigue trace of reinnervated EDL muscles taken from an animal with a bilateral P crush 3 (top trace) and 9 mm (bottom trace) away from the muscles is presented here.

B. An example of a fatigue trace of control (top trace) and operated (bottom trace) EDL muscles taken from an animal with unilateral P nerve crush 9 mm away from the muscle is shown here.
unilateral P nerve crush 9 mm away from were also established. These were 0.28 ±0.09 for reinnervated EDL muscles and 0.71 ± 0.03 (S.E.M.) for contralateral control EDL muscles. Using an unpaired t-test the difference between these two was found to be significant at p<0.05 level. These results are shown in Table 3.VI.

Figure 3.9 summarizes the results of Table 3.VI graphically. It can be seen from the Figure that EDL muscles reinnervated after P nerve crush 3 mm away from the muscles were more fatiguable than either their contralateral muscles which had had nerve injury 9 mm away from them or from reinnervated muscles from animals in group B which had had unilateral P crush 9 mm away. Interestingly, a Kruskall-Wallis one-way analysis of variance by ranks test (see Siegal, 1956) showed that the values in groups A and B were significantly different from each other at the p<0.05 level. Thus, each of the nerve injuries had changed the fatiguability of the EDL muscles significantly from normal values, but the extent of the change depended on the distance of the injury from the muscles.

3.3.9. Histochemical profiles of EDL muscles after bilateral P nerve crush 3 or 9 mm away.

Figure 3.10 shows cross-sections of 3 EDL muscles stained with succinic dehydrogenase. After bilateral P crush 9 mm away the reinnervated EDL muscle stained darkly and had small fibres (Figure 3.10, far right), matching its fatigue trace and fatigue index (see Figures 3.8 and 3.9). A cross-section of a normal EDL muscle taken from another animal matched for age and weight is shown on the extreme left of Figure 3.10 (courtesy M.B.Lowrie). This muscle showed the mosaic pattern of predominantly large pale glycolytic fibres
Table 3.VI.

Comparisons of mean ± S.E. fatigue indices of EDL muscles reinnervated after bilateral P crush 3 or 9 mm away from them and those of control and reinnervated EDL muscles after unilateral P crush 9 mm away from them are presented here. It was found that reinnervated muscles were less fatigue resistant the closer the crush was to the muscle. A paired t-test showed that there was a significant difference (p<0.01) between the fatigue indices of reinnervated muscles from the same animal after bilateral crush. An unpaired t-test used to test the difference between the fatigue index of reinnervated EDL and its contralateral control showed that this was significant at the 0.05 level. Furthermore an unpaired t-test performed on fatigue indices of reinnervated EDL muscles after P crush 3 mm away and those of control muscles showed that there was a significant difference at the 0.01 level. Finally, a Kruskall-Wallis one-way analysis of variance by ranks test showed that all the 4 groups presented in this table were significantly different from each other (p<0.05).
### Table 3.VI.

<table>
<thead>
<tr>
<th></th>
<th>Bilateral P crush</th>
<th>Unilateral P crush</th>
</tr>
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<tbody>
<tr>
<td>3 mm away</td>
<td>0.55 ± 0.08</td>
<td>0.71 ± 0.03</td>
</tr>
<tr>
<td>9 mm away</td>
<td>0.43 ± 0.06</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>Control uninjured 9 mm away</td>
<td></td>
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</table>
**Figure 3.9.**

This Figure summarizes table 3.VI graphically. Fatigue indices of reinnervated EDL muscles after bilateral P crush are shown on the left in A and those of control and reinnervated EDL muscles on the right in B. Hatched bars represent mean fatigue indices of reinnervated EDL muscles after P nerve crush 9 mm away from them. All four means are significantly different from each other.
Figure 3.10.
Examples of cross sections of EDL muscles reacted for succinic dehydrogenase (SDH) are shown in this figure. The darkly stained muscle fibres show high activity of oxidative enzymes. Normal EDL muscles have a characteristic mosaic pattern of large pale fibres with little SDH activity, interspersed with small darkly stained ones (see picture on extreme left labelled "Normal"). After P nerve crush 9 mm away from the muscles the muscle fibres stained darkly and homogenously (see picture on far right labelled "Far"). After P nerve crush 3 mm away from the muscles however, although the mosaic pattern was lost, some pale fibres were still seen (central picture, labelled "Near").
interspersed with small dark oxidative ones. In the centre is a cross-section of the contralateral EDL muscle (from the same animal whose right EDL is shown on the far right of the figure) reinnervated after a P nerve crush 3 mm away. Although most of the fibres seen were small and darkly stained, some larger paler fibres were still visible. It was found that when 10% of the muscle was sampled microscopically (400 fibres were counted in EDL muscles from 2 animals) 17% of the fibres although regrouped were pale. Since the oxidative capacity of a muscle is known to be an indicator of the fatiguability of muscle fibres (see Lowrie et al., 1982), it was reassuring to find that these two parameters were consistent with each other in reinnervated EDL muscles after bilateral P nerve crush 3 mm away from the muscles.

In conclusion then, it would appear from the results presented above that the shorter the period of denervation the better was the recovery of fast muscles after a neonatal nerve crush.
3.4 Discussion.

The results discussed in this chapter showed that the period of denervation of TA and EDL was shorter when the nerve was crushed close to the muscles (for example 3 mm away). It was also clear from the observations of behavioural recovery that there was no latent period during regeneration of the crushed nerves. Return of toe-spreading and dorsiflexion of the ankle were seen between 5 and 6 days after crush in the left leg in animals which had had their nerves crushed 3 mm away from TA and EDL (group A) and between 9 and 10 days on the contralateral side in the same animals where the LP nerve was crushed 9 mm away. Thus, the rate of regeneration of the crushed nerves was between 1.5 mm a day and did not change whether the crush was close to or far from the muscles. However, an added variable that must be taken into account for these experiments, was the growth of the animal. This would imply that the site of crush would also move further away from the muscles as the nerve was regenerating and would account for the unvaried difference of 4 days that was observed between the onset of recovery seen in muscles whose nerves were crushed 3 mm away or 9 mm away from them. Behavioral recovery was seen at the same time whether the P or sciatic nerves were crushed 9 mm away from the muscles no matter whether the animals had had unilateral or bilateral P or sciatic nerve crush.

These results were also in good agreement with those obtained in another study from this laboratory in which the sciatic nerve was crushed between 9 and 10 mm away from the muscles in 4 to 6 day old animals. Using the rate of advance of axon tips, the rate of regeneration of the injured nerves was found to be 1.6 mm per day.
The results obtained on the weights and tensions of TA and EDL (see Figure 3.2) showed that the closer the nerve injury was to the muscles the better was their resultant recovery. Thus, the recovery of the muscles was directly related to the period of denervation. Recent results from this laboratory (M.B. Lowrie, personal communication) have confirmed this finding by showing that delaying reinnervation of the fast muscles by crushing the sciatic nerve, one week after the initial crush inflicted at 5 days of age, also caused their increased impairment.

After a nerve crush 3 mm away from the muscles the recovery of muscle weights and tension was 50% to 60% of that estimated from normal weight matched animals (see Figure 3.5) whereas if the nerve was crushed 9 mm away from the muscles, the tension and weight of reinnervated TA and EDL was less than 50% of estimated normal. This was the case whether the nerve crush was unilateral and 9 mm away from the muscles or part of a bilateral crush experiment at the same distance away.

However, it was interesting to note that although the P nerve was crushed just 3 mm away from the muscles, the recovery of tension produced by reinnervated TA and EDL was just above half that estimated from weight matched control animals. Thus, even the short period of denervation that this injury produced was enough to disrupt the normal development of these fast muscles to permanently impair their chances of a complete recovery. A previous study (Brown et al., 1976) had shown that in new born animals, injury to the nerve just before its entry into the soleus muscle resulted in no death of the motoneurones supplying it. It was therefore thought that the muscles too recovered completely from the insult. Lowrie et al., (1982) later
showed that in rats unilateral sciatic nerve crush at 5 days after birth resulted in no loss of motoneurones supplying TA, EDL or the soleus muscles and that the latter muscle recovered its tension and weight and was 80% that of its contralateral control. But they found that the fast muscles, TA and EDL showed less than 50% recovery of tension and weight of their contralateral controls a value similar to that in the present expriments where the nerve was crushed 9 mm away from TA and EDL. Furthermore, these authors also found that the muscle fibre loss seen from TA and EDL occurred upon reinnervation (Lowrie and Vrbova, 1984). It was clear from the results of the study presented in Chapter 2 of this thesis that there was no difference in the time of arrival of regenerating axons to either fast or slow muscles and that even so, the fast muscles TA and EDL did not recover as quickly or as well as did the soleus. In fact, both TA and EDL showed a similar trend to that observed by Lowrie et al., (1984) in that they began to become impaired as reinnervation commenced. In a recent study the effect of the age of sciatic nerve crush on the muscle recovery was followed (Lowrie et al., 1987). It was found that the recovery improved when the injury was inflicted at later stages of postnatal life. Thus, after sciatic crush at 11 days reinnervated fast muscles recovered 65% to 75% of tension produced by their contralateral uninjured muscles. The recovery of the soleus muscle remained at 80% of its contralateral control. It is clear from the results presented there and those of previous studies that young fast muscles are vulnerable to disconnection from their motoneurones even if the period of separation is very brief.

One interesting finding of this study was the reduced fatigue resistance seen in EDL muscles after P nerve crush 3mm away. This
correlated well with the oxidative capacity of these muscles (refer to Figures 3.8 and 3.10). This result contrasted with the findings of Lowrie et al., (1987) who found that after unilateral sciatic nerve crush up to 12 days postnatally, the reinnervated EDL muscles were very fatigue resistant. However, histological examination of these muscles did show some pale fibres (Figure 4C Lowrie et al., 1987). One explanation for the results of the present study after P nerve crush 3 mm away from the muscles could be that the fatiguability of fast muscles may be different because the period of disconnection from their nerves was short. Alternatively, the difference may have been due to the crushing of the P nerve alone and not the whole sciatic. But this was found not to be the case. Fatigue indices of reinnervated EDL muscles after unilateral P crush at 5 days were found to be similar to those after whole sciatic crush at the same age (refer to Figure 3.9 and Lowrie et al., 1982;1987). However, the mean fatigue index of reinnervated EDL muscles after P nerve crush 9 mm away was greater than either that after unilateral P or sciatic crush where the respective nerves had been injured at approximately the same place.

Could the near normal fatigue index seen in reinnervated EDL muscles after P nerve crush 3 mm away be explained by less mismatch between the developing motoneurones and their immature targets? Navarrete and Vrbová (1980; 1983), found that the development of adult activity patterns in fast and slow muscles occurred during the first 2 weeks of life as locomotor patterns developed. Muscle activity was used as an index of motoneurone activity since it was known that the pattern was imposed by the latter (see Chapter 1, General Introduction). Thus, fast muscles such as EDL developed bursts of high frequency activity in response to movement. Slow muscles retained low
frequency tonic activity. Neonatal sciatic nerve crush permanently altered the development of the adult activity patterns in the reinnervated fast muscles (Navarrete et al., 1984). They showed an increase in activity which was 2 to 3 times that of their contralateral control muscles. In addition, they became highly responsive and continued to be active even when their antagonists were stimulated.

These results and those of other authors (Lowrie et al., 1982; 1987; Kashihara et al., 1987;) suggested that in developing neuromuscular systems not only was the target dependent on innervation by its motoneurones, but that the motoneurone too required constant interaction with its target. In the context of the results presented here, this reasoning would account for the loss of some muscle fibres (seen as a 40% to 50% decrease in muscle weight) after bilateral P nerve crush. It would also favour the near normal fatigue indices seen after P nerve crush 3mm away, since the distance that the regenerating axons had to travel was much reduced and consequently, the mismatch between the activity of motoneurones and their targets would be curtailed.

The increase in the fatigue resistance of adult fast muscles reinnervated after unilateral sciatic nerve crush during the first week of life was always accompanied by the absence of glycolytic fibres in them (see Lowrie et al., 1982; 1984; 1987). Since this type of injury resulted in a substantial loss of muscle fibres, and these were lost upon reinnervation, it would follow that those fibres that were lost were fated to become large glycolytic ones. In the present study, the loss of large pale muscle fibres, low in oxidative activity was seen in reinnervated adult EDL muscles which had had a P nerve
crush at 5 days 9mm away from the muscles. However, those muscle fibres which had had a P nerve crush 3mm away did show some pale fibres (see Figure 3.10). Although these were not in the characteristic mosaic pattern of distribution of slow fibres seen in normal adult muscles, they nevertheless suggested that some large fibres had been preserved. Once again, this finding may be explained due to the shorter period of denervation and reduced mismatch between young motoneurones and their targets. In addition, it could be that this reduced "mismatch" could be due to the decreased mobility of these animals with bilateral nerve crush in comparison to those with unilateral nerve crush.

In conclusion then, the results presented in this chapter have clearly shown that young fast muscles require constant interaction with their motoneurones for their normal development. In addition, the results have shown that temporary denervation at a critical period of development results in the impairment of muscle function and more importantly, that recovery of function upon reinnervation is related to the length of time that they are left denervated. That motoneurones are also affected by neonatal nerve injury because of the temporary absence of retrograde influences from the muscle has been shown by results of previous studies (see Navarrete et al., 1984, Vrbová et al., 1985). It would be interesting to investigate the reorganisation of synaptic inputs that may occur as result of bilateral nerve injury inflicted neonatally. An attempt has been made to investigate the effects of unilateral nerve crush on the reflex activity of the reinnervated fast muscles and the results of these experiments are discussed in Chapter 4.
CHAPTER 4.
Long lasting modifications of reflexes induced by nerve crush.

4.1. Introduction.

The results of the previous chapters and those of other studies showed that the effects of temporary disruption of interaction between nerve and muscle during early postnatal life left the fast muscles permanently impaired. In this chapter, the effects of a similar injury in early postnatal life on the alterations of reflex activity that resulted as a consequence of neonatal nerve injury are studied.

The importance of constant contact with the target muscle for the normal development and survival of motoneurones that supply it is well known in avian and mammalian embryos (Hamburger, 1975; Oppenheim, 1988) and in new born mammals, so that even temporary disruption results in the loss of more than half of these (Romanes, 1946; Zelená and Hnik, 1963; Kashihara et al., 1987; Lowrie et al., 1987). However not much information exists on the effects of this temporary separation of the motoneurone from its target on the development of functional connectivity of the surviving motoneurones. Evidence from electromyograms of reinnervated fast muscles after sciatic nerve crush at birth showed that there is a permanent alteration in their activity pattern during spontaneous locomotion when they were compared with normal fast muscles (Navarrete and Vrbová, 1984). A consequence of the increased functional activity of reinnervated fast muscles was the increase in their fatigue resistance and oxidative capacity (Lowrie et al., 1982; Navarrete and Vrbová, 1983). Interestingly, reinnervated fast muscles showed reduced fatigue resistance and oxidative enzyme activity if the nerve injury was made close to them so that the duration of denervation was reduced (see
Chapter 3). In addition, previous evidence showed that changes of activity patterns and properties of muscles were not seen after nerve injury in animals older than 12 days of age (Navarrete and Vrbova, 1984; Lowrie et al., 1987). Thus modifications of activity and properties only occurred after nerve crush during early postnatal life and could probably be minimised if the period of denervation were reduced (Chapter 3, this thesis). Sciatic nerve lesions in newborn animals not only affect motoneurones but also interrupt the sensory afferent input to the spinal cord from muscle and skin targets innervated by this nerve. It is known that following nerve lesions in neonatal animals a significant number of neurones in the dorsal root ganglia die so that at least part of the segmental afferent input to the motoneurone is permanently lost (Zelená and Hník, 1963; Bondok and Sansone, 1984; Yip et al., 1984). Thus, it is possible that peripheral nerve injury during early stages of development may result in a permanent alteration of the synaptic input to the motoneurones. Whether surviving or undamaged sensory neurones are able to expand their projection to occupy the territory normally occupied by the neurones that die is presently unknown. However, some experiments indicate that after neonatal nerve injury intact sensory fibres are able to expand their projections (Fitzgerald, 1985). Moreover, synaptogenesis in the spinal cord is still in progress during the first 2 weeks after birth (Stelzner, 1982) and it may be that regenerating motoneurones are more likely to receive inputs than those cells that have already terminated their growth. Since the vast majority of synaptic inputs to motoneurones is mediated via interneurones, an indication of abnormal synaptic drive to the injured
motoneurones would be provided by changes in polysynaptic reflexes.

In the results described here the polysynaptic reflex responses of reinnervated EDL muscles evoked by stimulation of ipsilateral and contralateral branches of the sciatic nerve are discussed.
4.2 Methods.

Surgery.

In 12 rats aged 0 to 5 days (neonatal crush) and in 6 rats aged 2 months (adult crush) the right sciatic nerve was exposed in the thigh and crushed using fine watchmakers forceps (see Methods in Chapters 2 and 3). In three 5 day old animals, only the right peroneal nerve was crushed in the thigh. These operations were performed under either ether (neonatal crush group) or chloral hydrate (4.5% i.p., 1ml/100g body weight; adult crush group) and using sterile precautions. Following the operation, the motor nerves returned to their original muscles and reinnervation was complete after 6 weeks (Lowrie et al., 1982;1987; Chapter 3, this thesis).

In 2 animals, 3 to 6 months after sciatic crush at birth, teflon coated stainless steel electrodes were implanted into extensor digitorum longus (EDL) muscles on both sides using the method described by Hnik et al., (1988). The operation was carried out under chloral hydrate anaesthesia (as above for adult crush group) and using sterile precautions. Electromyographic recordings were carried out daily, for upto 2 weeks after the operation in the freely moving animals once they had recovered from the anaesthetic.

Three to six months after the operation, in the final experiment, the remaining animals were anaesthetised using ether, the trachea cannulated and a cannula inserted into the right jugular vein for intravenous administration of the steroid anaesthetic alphaxolone/alphadalone (Saffan, Glaxo; initial dose 3.5 mg/kg). Throughout the experiment supplementary doses of anaesthetic were given. The choice of this particular steroid anaesthetic was made because it was easily administered intra-venously, produced reliable muscle relaxation and
anaesthesia. Unlike chloral hydrate, which causes loss of reflex activity, and is effective as an analgesic only in large doses, the combination of alphadalone/alphaxalone can be administered safely as an infusion for up to 10 hours without the development of tachyphylaxis, tolerance or cumulative effects developing (see Green, 1979).

Recording.

**EMG records from freely moving animals.**

These were obtained by putting the signal through a preamplifier (Neurolog NL 104A.C. Preamplifier), displayed on a storage oscilloscope (Tektronix 5113) and recorded on an FM tape recorder (Racal 4DS).

**Recording of reflex activity in acute experiments.**

In anaesthetised animals, the tendons of the extensor digitorum longi muscles on the operated and unoperated sides were dissected. Two teflon-coated stainless steel wires 75 um in diameter were bared at the tip and sutured into the bellies of both EDL muscles so as to record the electromyogram (EMG) from each muscle.

The peroneal (P), tibial (T) and sural (S) nerves were dissected in the thigh. The skin and surrounding muscles were sutured to special holders in order to make a paraffin pool. The tendons of both EDL muscles were connected to strain gauges (Devices Dynamometer UFI Transducer). Individual nerves were stimulated via bipolar silver electrodes by square wave pulses of 0.05 ms duration and of varying intensity. The reflex responses evoked by stimulation of each nerve were quantified by expressing the reflex tension as a percentage of the supramaximal twitch tension obtained by stimulation of the motor nerve.
4.3 Results.

4.3.1 The effect of sciatic nerve crush at birth on gait.

EMG recordings taken from the reinnervated and control EDL muscles in 3 to 6 month old rats showed an overall increase in the activity of the former. The pattern of activation of the two muscles was then compared.

Figure 4.1 shows that the control muscle was active mainly during the swing phase of the leg. This was true for the operated muscle too, but, in addition, EMG activity was seen during the stance phase and was synchronous with that seen during the swing phase of the control limb (see also Navarrete and Vrbova, 1984). Thus, it appeared that regenerated flexor motoneurones received excitatory inputs at appropriate and inappropriate times. To analyse further the origin of inappropriate inputs, responses of reinnervated and control EDL muscles to stimulation of various branches of injured and uninjured sciatic nerves were examined.

4.3.2 Effects of nerve injury in neonatal animals on reflex responses.

Reflexly elicited EMG activity was recorded from EDL muscles on both sides by stimulating various branches of the sciatic nerve. The experiments were carried out on animals that had their sciatic nerve injured at 0-6 days of age.

Figure 4.2 shows records from one such experiment. EMG responses were elicited by stimulation of either the operated (A) or the control (B) peroneal (P) nerve. It can be seen that stimulation of the P nerve on the reinnervated side (Figure 4.2 A) elicited first a response from the (operated) EDL muscle, which was due to direct activation of motor fibres in the nerve. This was followed by an
The Figure shows EMG records from an adult animal 3 months after sciatic nerve crush at 5 days. The animal was ambulating in its cage. The top trace (op) was that of the reinnervated EDL muscle and the bottom was its contralateral control. The control muscle was active only during the swing phase of its step cycle. The reinnervated muscle on the other hand was active during both the swing phase and the stance phase of its step cycle.
Op

Con

0.25mv

100msec
Figure 4.2.

EMG responses recorded s.multaneously from both EDL muscles were elicited by stimulation of the regenerated (A) or control (B) peroneal (P) nerve. The stimulus is indicated by the arrows. The sciatic nerve on the operated side (op) was crushed at birth. Note that stimulation of the control (con) nerve in (B) elicited a crossed-reflex response on the contralateral operated (op) EDL muscle.
ipsilateral reflex response of short latency. In contrast, stimulation of the control P, in addition to activating the control muscle by its motor nerve, also elicited a reflex response from the contralateral reinnervated, operated muscle (Figure 4.2 B). This reflex response had a latency of 16.5 ± 2.13 ms SEM (N=8) and was first elicited at a stimulus intensity similar to that needed for just suprathreshold stimulation of motor fibres in the P nerve, thus suggesting that the sensory fibres involved in this reflex response were of low threshold. This result indicated therefore, that after peripheral nerve injury during the early postnatal period, EDL motoneurones had an enhanced response to stimulation of the homonymous nerve on the contralateral side.

It could be argued that the large contralateral response of the reinnervated muscles to stimulation of the homonymous control nerve was the usual reflex response and that the relatively diminished reflex response on the control side to stimulation of the neonatally injured nerve could be due to a permanent impairment of sensory nerve fibres resulting from this injury. Therefore, further experiments were carried out to exclude the possibility that damage to sensory nerves due to the sciatic nerve crush was responsible for the discrepancy of reflexes elicited by stimulating the operated or unoperated nerves. In 3 animals the P nerve alone was crushed at 0-5 days after birth and all other branches of the sciatic were left intact. The results shown in Figure 4.3 A show that stimulation of the intact T on the operated side elicited a strong ipsilateral reflex, but no response on the contralateral side. In contrast, stimulation of the control MP nerve elicited an ipsilateral as well as a strong reflex response in the reinnervated EDL muscle (Figure 4.3 B). Similar results were obtained
Figure 4.3.
Reflex responses were elicited by stimulation of the tibial (T) nerve in an animal in which the peroneal (P) nerve was crushed at 5 days of age. In (A) stimulation of the uninjured T on the operated side produces only an ipsilateral reflex response. By contrast, stimulation of the T on the control side (B) elicited a flexor reflex response in the ipsilateral (con) EDL muscle followed by a crossed-reflex response in the contralateral (op) EDL.
by stimulating the S nerve. Thus, an increase in the reflex response of the reinnervated muscle was present, even though the heteronymous nerve used to elicit the reflex was undamaged in the original operation.

The strength of "reflex" responses of both control and reinnervated muscles evoked by stimulation of ipsilateral and contralateral branches of the sciatic nerve, was estimated by expressing the "reflex" tension developed by the muscle in response to sensory stimulation as a percentage of the maximum twitch tension produced by each of the muscles when its own motor nerve was stimulated. Table 4.1 summarizes the results expressed in this way. It shows that the reinnervated muscles developed greater reflexly elicited tension than that produced by uninjured control muscles.

Another indication of the increased responsiveness of the reinnervated EDL muscles to sensory stimulation was the occasional presence of an afterdischarge following a reflex response. This is illustrated in Figure 4.4. A single stimulus to the injured P nerve was followed by repetitive motor unit activity in the reinnervated EDL muscle. This was never seen in uninjured muscles or those that had been injured in adulthood.

In order to compare reflex responses from animals with neonatal nerve injury to those of normal unoperated ones responses to stimulation of the same branches of the sciatic nerve were examined in 3 normal adult animals. Stimulation of the P nerve elicited a small and variable reflex response (0.76± 0.76 of the maximum twitch tension) even when supramaximal stimulus intensities were used. The threshold for an ipsilateral reflex response was between 0.3 and 0.5
Table 4.I.

Reflex tension produced in response to stimulation of various branches of the sciatic nerve expressed as a percentage of the total twitch tension produced by the muscle. Animals with neonatal sciatic nerve crush. Results are expressed as mean + standard error of the mean. The number of animals was 10. Abbreviations used are as follows: P = peroneal nerve, T = tibial nerve, S = sural nerve.
Table 4.1

<table>
<thead>
<tr>
<th>Nerve Stimulated</th>
<th>Reinnervated Muscle</th>
<th>Control Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operated P</td>
<td>*</td>
<td>No response</td>
</tr>
<tr>
<td>Unoperated P</td>
<td>17+4.34</td>
<td>*</td>
</tr>
<tr>
<td>Operated T</td>
<td>9.96+4.13</td>
<td>2.54+1.68</td>
</tr>
<tr>
<td>Unoperated T</td>
<td>11.25+4.75</td>
<td>1.08+0.74</td>
</tr>
<tr>
<td>Operated S</td>
<td>3.02+1.79</td>
<td>1.31+0.76</td>
</tr>
<tr>
<td>Unoperated S</td>
<td>4.98+2.73</td>
<td>0.12+0.12</td>
</tr>
</tbody>
</table>

* The reflex response could not be assessed due to the motor response when the muscle's own nerve was stimulated.
Figure 4.4.
Illustration of repetitive motor unit activity after a single stimulus to the peroneal (P) nerve from a reinnervated EDL muscle following sciatic nerve injury at birth.
volts. Stimulation of the T nerve elicited a good ipsilateral response which was between 8% to 14% of the maximal twitch tension. Only a weak contralateral response was apparent. It was difficult to measure as a tension response and could only be seen as asynchronous EMG. Stimulation of the S nerve produced small ipsilateral and contralateral responses that never exceeded 2% of twitch tension. Thus, responses of normal animals were comparable to those of the reflexes recorded from the unoperated side of the experimental animals.

4.3.3 Effect of nerve injury in adult animals on reflex responses.

The results discussed above showed that nerve injury during the first week of life altered the reflex responses of reinnervated EDL muscles. It could be argued that it was the injury per se that induced the changes seen, and that this effect of injury may not be unique to neonatal animals. Therefore, in six 2-month-old animals the right sciatic nerve was crushed and their reflex responses studied 3 months later. Tension and EMG responses were recorded simultaneously from both EDL muscles as described above.

The results are summarised in Table 4.II. Stimulation of the uninjured P nerve elicited a small response in the reinnervated muscle in 4 out of 6 experiments. Stimulation of the regenerated P nerve also elicited a reflex response from the uninjured muscle. This was different from the results obtained in control animals or in animals that had had a neonatal nerve crush, in which the unoperated muscles showed no response to stimulation of contralateral injured nerves (compare Tables 4.I and 4.II).

Stimulation of the T and S nerves produced good responses both as
Table 4.II.
Reflex tension produced in response to stimulation of various branches of the sciatic nerve expressed as a percentage of the total twitch tension of the muscle. Animals with sciatic nerve crush in adulthood. Results are expressed as mean + standard error of the mean. The number of animals was 6. Abbreviations used are as follows: P = peroneal nerve, T = tibial nerve, S = sural nerve.
**Table 4.II**

<table>
<thead>
<tr>
<th>Nerve Stimulated</th>
<th>Reinnervated Muscle</th>
<th>Control muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operated P</td>
<td>*</td>
<td>7.91 ±4.13</td>
</tr>
<tr>
<td>Unoperated P</td>
<td>3.65±2.59</td>
<td>*</td>
</tr>
<tr>
<td>Operated T</td>
<td>8.18±3.04</td>
<td>3.43±1.94</td>
</tr>
<tr>
<td>Unoperated T</td>
<td>6.2±4.8</td>
<td>6.84±2.40</td>
</tr>
<tr>
<td>Operated S</td>
<td>4.13±2.18</td>
<td>3.6±1.61</td>
</tr>
<tr>
<td>Unoperated S</td>
<td>5.95±2.43</td>
<td>5±2.44</td>
</tr>
</tbody>
</table>

* The reflex response could not be assessed due to the motor response when the muscle's own nerve was stimulated.
asynchronous EMG and tension responses in 3 out 6 animals studied. These reflex responses were always greater on the ipsilateral side. There was no appreciable difference between responses of the control or operated muscles in these animals unlike in those seen in animals which had sustained a neonatal nerve crush. Increasing stimulus intensity to supramaximal that of threshold intensity also had no effect on the size of these reflex responses. Thus, it would appear from these results that the reflex responses of regenerated EDL motoneurones injured in adulthood remain unaltered.
Discussion.

The results described in this chapter have shown that temporary interruption of contact between the motoneurones and their target muscle during early postnatal life led to a permanent enhancement of spinal reflexes involving the neonatally injured motoneurones. This was most apparent for responses elicited by stimulation of contralateral branches of the sciatic nerve which usually evoked very small crossed-reflex responses in ankle flexor muscles of normal animals or uninjured side with neonatal nerve injury.

The reason why nerve crush in young animals should produce long lasting alteration in reflex responses still remains unclear. One possibility could be that the observed changes may have been due to a permanent alteration of synaptic inputs to the injured motoneurones. In adult animals, peripheral nerve lesions (crush or axotomy) have been shown to result in a temporary decrease in the amplitude of the monosynaptic excitatory post synaptic potential (EPSP) suggesting an alteration of the primary afferent input onto motoneurones following disconnection from their targets (Eccles et al., 1959; Kuno et al., 1970; Mendell, 1984). These changes in synaptic inputs to motoneurones were restored to normal 8 weeks later, once reinnervation after sciatic nerve crush was complete (Gallego et al., 1979).

Preliminary results using an in vitro model of a neonatal spinal cord hind-limb preparation have shown that presumptive fast or slow motoneurones had marked afterdepolarization and afterhyperpolarization which decreased with age (Navarrete et al., 1987). In addition, stimulation of the tibial nerve not only produced a monosynaptic reflex EMG response in the soleus muscle but also a reflex EMG response in its antagonist the ankle flexor tibialis anterior 1 to 5
milliseconds later. This co-activation was age-dependent so that, at postnatal day 3, it occurred in all preparations but by postnatal days 8 to 10 it had dropped to 33% (Navarrete et al., 1988). Since this early postnatal period is also known to be a time for active synaptic reorganization to occur (Conradi & Ronnevi, 1975), a disruption in the afferent and efferent inputs by sciatic nerve crush during this period would be bound to result in the alteration or indeed perhaps the maintenance of original synaptic connectivity.

In newborn animals sciatic nerve crush leads to the death of large numbers of sensory neurones and motoneurones (Romanes, 1946; Zelena & Hnik, 1963; Bondok & Sansone, 1984; Yip et al., 1984; Lowrie et al., 1987). The precise extent of motoneurone death depends on the age of the animal and the type of injury (see Romanes, 1946; Lieberman, 1974; Schmalbruch, 1984; Lowrie et al., 1987). The loss of sensory neurones could result in the permanent loss of synaptic inputs to spinal neurones (including motoneurones) onto which they normally project, and it may be that the vacated synapses become occupied by other inputs.

The possibility that surviving or undamaged primary afferent neurones may be able to expand their monosynaptic connections to motoneurones to occupy the synaptic sites vacated by the neurones that die has been considered in several previous studies. Eccles, Eccles and Shealy (1962) studied the effects of injury to nerves of the deep peroneal and medial gastrocnemius muscles inflicted in very young kittens on the monosynaptic activation of their motoneurones by afferent inputs from their own and other afferent nerves. In this way they intended to test the possibility of non-specific sprouting from
intact afferents of uninjured nerves. Several months after reinnervation, the size of the EPSP elicited by stimulation of the previously injured nerve was reduced (a fact attributed to the degeneration of primary afferent neurones) but no change in the extent of monosynaptic activation by primary afferent neurones in the intact nerves was observed. This was consistent with later observations on chick embryos where after partial deafferentation produced by neural crest lesions, there is little evidence for aberrant monosynaptic connections (Eide et al., 1982).

Results from a recent study indicate that following medial gastrocnemius nerve crush in one-day-old rats, stimulation of Ia afferents in the injured nerve evoked monosynaptic EPSPs only in 40% of medial gastrocnemius motoneurones sampled. In addition, even when an EPSP was elicited, its size was very much reduced (Miyata et al., 1986).

Thus, nerve injury during the perinatal period seems to result in a permanent reduction of monosynaptic inputs with no replacement of these afferents from other intact nerves. The only report of aberrant monosynaptic connections to motoneurones is that by Eccles et al., (1960), who found that following cross-reinnervation of peroneal muscles by the gastrocnemius nerve some peroneal motoneurones become monosynaptically excited by afferents in the lateral gastrocnemius nerve. The majority of the existing reports thus show an overall decrease of monosynaptic inputs after neonatal nerve injury.

However, only a small part of the total synaptic input to motoneurones is monosynaptic and the vast majority of it is mediated via interneurones (Baldissera et al., 1981). The present results show alterations of these polysynaptic spinal reflexes. Two possible
explanations for the present findings are: a) that some interneurones that normally project to motoneurones may sprout to occupy synaptic space vacated by lost projections or, b) that synaptic connections that are formed by these neurones during early synaptogenesis may fail to retract after birth. Examples of such plasticity have been demonstrated in various regions of the developing and adult nervous systems (Cotman et al., 1981; Mendell et al., 1984; Purves & Lichtman, 1985). The finding that reflex responses evoked from contralateral afferents are markedly enhanced compared to normal indicates that one such input could arise from commissural interneurones which project to contralateral motor nuclei and which are known to be involved in crossed reflexes (Harrison et al., 1986). It may be hypothesized that if the mechanism by which restriction of reflexes is achieved during development involves retraction of expanded connections formed in early stages of ontogenesis (Saito et al., 1979) then the enhancement of the reflex pattern seen after neonatal nerve injury may be a remnant of the neonatal reflex pattern.

An additional factor responsible for the present findings may be a change in motoneurone properties following neonatal nerve injury. In some experiments, particularly when the sciatic nerve was crushed a few hours after birth, a single stimulus to the P nerve evoked a prolonged afterdischarge (see Figure 4.4). It is unlikely that such longlasting activity may originate in the peripheral nerve since it was readily abolished by increasing the level of anaesthesia. On the other hand, it may be possible that after neonatal nerve injury some motoneurones become and remain more excitable than normal. Several parameters related to the excitability of the motoneurone (i.e., size,
passive and active membrane properties) continue to develop after birth (Kellerth et al., 1971; Fulton & Walton, 1986) and their full maturation may depend upon uninterrupted synaptic contact with the target. Other evidence also indicates that after sciatic nerve crush in the rat the cell body size of the largest EDL motoneurones (which are known to innervate fast-fatiguable muscle fibres) remains permanently reduced (Lowrie et al., 1982). Thus, it appears that at least some properties of the motoneurone itself may be altered after neonatal nerve injury.

The enhanced reflex responsiveness of the injured flexor motoneurones is also reflected in their everyday function as seen by their increased overall activity and their abnormal recruitment during locomotion (see Figure 4.1; Navarrete et al., 1984; Vejsada et al., 1987).

The experiments described in the next chapter were an attempt to increase the chances of the normal development of motoneurones after a neonatal nerve lesion at birth using various agents.
Chapter 5.
The effect of reducing the Calcium concentration at the site of neonatal nerve crush on the survival of motoneurones.

5.1 Introduction.

In this thesis some of the responses of developing motoneurones and muscles to axon injury during early perinatal development have been studied. This Chapter will consider motoneurone death which occurs as a result of sciatic nerve crush at birth and attempt to prevent motoneurone loss. The dependence of motoneurone survival on continuous interaction with the target will also be considered.

In the spinal cord of vertebrate embryos motoneurones are the first cells to migrate to their appropriate positions (Cajal, 1928). Muscle cells develop independently from mesenchymal cells to become myoblasts and myotubes. It is at this stage that the first exploring axons reach their targets and begin to make synaptic contact with the myotubes. It is at this time that motoneurones begin to depend on their targets for their survival (for review see Purves and Lichtman, 1985). Thus, for example, limb bud removal in chick embryos results in the degeneration of the entire ventral horn if the axons of the already formed motoneurones are prevented from contacting their targets although motoneurone proliferation continues in up to 5 day old embryos.

However, even in the presence of limbs, many motoneurones are known to die during normal development and this happens when the first peripheral connections are made (Hamburger, 1975). Similar cell death has been reported in several other embryos such as mouse, rat and amphibian and it always coincides with the appearance of neuromuscular contacts and spontaneous movement (Hughes, 1961; Chu-Wang and
Oppenheim, 1975; Oppenheim and Chu-Wang, 1977). It was thought that those cells that die arrived at targets that were already innervated and therefore failed to make connections. Hamburger and Keefe (1944) showed results consistent with this idea when they transplanted an additional wing in chick embryos and found that the number of motoneurones that survived was greater than normal. By the same token the number of neurones that died could be increased after partial removal of the peripheral target (Hughes, 1968; Hamburger, 1975).

Based on these results Hamburger and Levi-Montalcini (1949) thought that the target released a "trophic" factor which guided axons to the receptor sites on it. Thus, the motoneurones that would survive would be those which succeeded in competing for the limited sites existing on the muscle.

Pittmann and Oppenheim (1978) thought that blocking the post synaptic membrane of the target with α-bungarotoxin would result in the death of all motoneurones in the chick embryo because no "trophic" factor would be available to them. But their results were totally opposite to their expectation in that more motoneurones survived than would have done normally. However, this was only true if the neuromuscular block was introduced before the onset of naturally occurring cell death. Several authors have reported similar results in embryos of other species (Laing and Prestige, 1978; Olek and Edwards, 1978; Oppenheim and Majors-Willard, 1978; Creazzo and Sohal, 1979a;). Thus, cell death has been found to be regulated partly by target size and partly by neuromuscular activity.

The period of dependency of the motoneurone on interaction with its target extends into early postnatal life. Disconnection from the
target at this stage results in motoneurone death. It is generally accepted that the muscle provides the motoneurone with retrograde influences which affect its metabolic state and sustain it during development when it is susceptible to death (Hamburger and Oppenheim, 1982).

In the rat, the time at which motoneurones are susceptible to loss of synaptic connections extends to the first week of postnatal life. Thus, axotomy or sciatic nerve crush at birth results in the death of 70% of the motoneurones in the ventral horn (Romanes, 1946; Beuker et al., 1951; Zelenci and Hnik, 1963; McArdle and Sansone, 1977; Schmallbruch, 1984; Lowrie et al., 1987).

Similar injuries made in adult animals do not result in motoneurone loss and it has been found that the older the animal the more likely are motoneurones to survive after axotomy or nerve crush (La Velle and La Velle, 1959; La Velle, 1964).

An explanation for the dependence of the young motoneurone on its target may come from studies on the differences between reactions of young and adult axons to axotomy or crush. Romanes (1946) and later others (see Lieberman, 1971; 1974) suggested that the vulnerability of young motoneurones was a result of the injury to their axons. Some studies do show a correlation between neuronal reaction to injury and the degree of maturation as judged by cytomorphic criteria. Thus, neurones whose axons had been injured at birth did not show reversible chromatolysis as did adult ones. Instead, they just disintegrated, degenerated and died (La Velle and La Velle, 1959; La Velle, 1964).

It has been suggested that young motoneurones die because they have not yet acquired the metabolic capability to regenerate and are unable to cope with the increased metabolic demands of axonal
regeneration. Watson (1969) has suggested that chromatolysis represents the reorganization of a neurone from a transmitting to a growing cell. So, when a young neurone is injured it may still be in a growing mode. It may be that in this condition it is sensitive to changes in the ionic microenvironment at its terminal end, the neuromuscular junction. These changes may be associated with transmission and may have a retrograde influence on motoneurone differentiation during the process of elimination of polyneuronal innervation (O'Brien et al., 1983; Connold et al., 1986). Whatever the explanation, it has been shown that it is vital for the young motoneurone to maintain contact with its target.

At this point it would be worth considering some of the changes a neurone undergoes as it switches from a growing to a transmitting cell. Evidence comes mainly from neurones in culture. The transition between a growing to a transmitting mode can be induced by making the tip of the elongating axon or the growth cone contact a muscle cell. Arrest of growth and an increase in transmitter release is immediately seen (Moo Ming Poo, 1988). This event may be induced by simple biophysical changes of the microenvironment at the nerve ending. Thus, when many growth cones of a single neurone start transmitting, the cell body may be induced to increase the synthesis of choline acetyl transferase (ChAT), which is the enzyme responsible for the production of the neurotransmitter acetyl choline (ACh) (Watson, 1969). It is thus that the target may induce the motoneurone to alter its phenotype. Recent results on sympathetic neurones indicate that for the development of their dendrites, interaction with their targets is vital (Voyvodic, 1987).
Several studies in the rat have shown that during early postnatal life interaction between young motoneurones and their targets induces a change in their characteristics and that if interaction is prevented at this period, it may be fatal to the motoneurones (Romanes, 1946; Zelena and Hnik, 1965; McArdle and Sansone, 1977; Buris, 1987; Kashihara et al., 1987; Lowrie et al., 1987). The nature of the change in motoneurones and muscles that occurs as a result of early postnatal interaction between them may be connected with the transition of the motoneurone from a growing to a transmitting cell.

The ionic environment around young injured axons may influence its regeneration. Several recent studies on growth cones of neurites in culture have shown that the regulation of calcium seems to play a central role in the extracellular environment with regard to growth and elongation (see Kater et al., 1988). In general, the results of these studies lead to one hypothesis: if calcium falls below an optimal level or rises significantly above it, growth cone motility and neurite outgrowth are inhibited. Experimentally evoked action potentials in some neurones in culture, completely inhibit growth cone development (Cohan and Kater, 1986). These action potentials have been shown to increase intracellular calcium in some neurones (Bolsover and Specter, 1986). Evidence that this calcium came from the extracellular environment of growth cones via calcium channels in the membrane and that it completely blocked growth cone activity in helisoma neurones was confirmed by experiments in which calcium channel blockers such as cobalt, lanthanum or cadmium were used (Cohan et al., 1987; Mattson et al., 1987).

Anglister et al., (1982) using pharmacological agents have shown that there is a relationship between voltage dependent calcium
channels and neurite outgrowth. Thus, when voltage dependent calcium channels are opened, calcium enters locally in response to any stimulus that depolarizes the growth cone sufficiently, suggesting that the major determinant of growth cone motility (the growing mode, in the case of the regenerating axon tip) or stability (transmitting mode when the axon has reinnervated the target) is the membrane potential (for review see Kater et al., 1988).

The link between the in vitro studies of growth cones and neurites and the growth cones of young regenerating axons in vivo maybe just these voltage dependent calcium channels. Injury to a nerve cell can cause leakage of potassium from the cut or crushed end of axons into extracellular space. Excess external potassium, if not buffered, is known to affect the excitability of nerve cells and eventually lead to their destruction (see Svoboda et al., 1988). The excess potassium in the extracellular space may open voltage-gated calcium channels raising its intracellular concentration. This would prevent some growth cones from elongating. The axons would then become unable to reach their targets and thus, may enhance cell death.

Consistent with this possibility Campenot (1983) found that sympathetic nerves did not grow or regenerate in the presence of increased potassium. This would be compatible with the idea that motoneurone death after neonatal nerve crush could be aggravated by the presence of high potassium.

The experiments described in this chapter explore such a possibility. Using the calcium chelating agent 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) alongside the site of crush of the sciatic nerve of new born rats, the possibility was tested of
enhancing the chances of survival of the motoneurones supplying the soleus muscle.
5.2 Methods

5.2.1. Surgery.

All surgery was performed on new born albino Wistar rats using ether anaesthesia and sterile precautions. An incision was made in the right thigh and the sciatic nerve was exposed and crushed above the popliteal fossa with a pair of fine Watchmakers forceps. The incision was then sutured and the animals were allowed to recover.

Three to 4 days later, some animals were anaesthetised with ether and a second operation was performed using sterile precautions. The original incision was reopened and a small 1 mg silicone rubber strip containing either 238 μg BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, BDH) or 9 μg (0.9%) NaCl (sodium chloride) was placed alongside the sciatic nerve at the site of the nerve crush. The incision was resutured and the animals allowed to recover.

5.2.2. Preparation of silicon rubber strips.

The implant strips were prepared as follows: a small quantity of either BAPTA or NaCl (in powder form) was weighed into a sterile Petri-dish and a calculated quantity of rubber solution (Dow Corning 3140, non-toxic) was added. The powder was thoroughly mixed with the silicone rubber and was allowed to set for 8 hours. When dry, small strips (3 mm x 1 mm x 0.5 mm, weighing 1 mg) were cut from the flexible piece of rubber. The approximate amount of compound per strip was 238 μg of BAPTA and 9 μg of NaCl. (see Connold et al., 1986).

5.2.3. Release of the experimental compound from the silicon rubber strip.

Previous (unpublished) results have shown that the release of calcium chelating agents from the silicone rubber strips continues for at least 3 days, about 35% of the total amount being released during
the first 24 hours. Further experiments were carried out as an in vitro model using the radioactive analogue of BAPTA, QUIN 2 H^3 (Connold et al., 1986; Connold, 1989).

5.2.4. Injection of Horseradish Peroxidase.

When the animals were at least 6 to 8 weeks old, they were prepared for localisation studies of the soleus motor pool. The body weights of the rats ranged from 175 to 500 grams. In general, muscle weights of hindlimbs in rats have been shown to correlate well with body weight (see Figure 5.1). Motoneurones were identified by retrograde labelling with horse radish peroxidase (HRP). The amount of HRP injected into each of the soleus muscles was calculated according to their body weight so that 1 ul of 15% HRP solution was given for each 25 mg of estimated muscle weight.

The 15% HRP (SIGMA TYPE VI) solution was made up in sterile 0.9% NaCl for injection into the muscle. The animals were anaesthetised with chloral hydrate (4.5% 1 ml per 100g body weight i.p.) and the HRP injections made using sterile precautions. A longitudinal incision was made on the dorsolateral aspect of each leg and using a microsyringe (Hamilton), the HRP injected directly into the soleus muscles of each leg about 5 mm distal to the entry of its nerve. The amount of HRP injected into the soleus muscles of animals which had sustained sciatic nerve crush at birth was adjusted to one third to one half of their estimated muscle weights since these muscles were smaller than their contralateral controls. In all animals, care was taken to ensure that minimal leakage of HRP occurred by injecting it very slowly while gradually withdrawing the needle. When spillage occurred it was immediately mopped up with cotton wool and the area was rinsed with
Figure 5.1.

In this Figure the correlation between body weight (x-axis) and muscle weight (y-axis) of Soleus muscles in normal adult animals is shown. A regression line was drawn and was used to estimate the amount of horse radish peroxidase to be injected into control muscles of experimental rats.
sterile saline. The incisions were then closed and the animals allowed to recover. They were allowed to survive for 36 hours.

5.2.5 **Perfusion and preparation of the spinal cord.**

Thirty six hours after the HRP injection, the animals were re-anaesthetised using 4.5% chloral hydrate injected intraperitoneally, and then perfused through the heart. Each animal was first perfused briefly with 0.9% saline solution to remove the blood and then with a fixative made up of 2.5% glutaraldehyde in Millonig's phosphate buffer, (pH 7.3) for approximately 30 to 40 minutes. About 1 ml of the fixative was used per gram of body weight.

The lumbar region of the spinal cord was carefully dissected out and placed in a small volume of the fixative and postfixed at 4°C for 4 hours. The spinal cord was then transferred to a 30% sucrose + Millonig's phosphate buffer (pH 7.3) and stored overnight at 4°C.

A section of the spinal cord was then cut just between segments L2 and L6 under a dissecting microscope. The segments were identified by the exit of their ventral roots and the position of dorsal root ganglia. It was between segments L4 and L5 that the soleus motoneurone pool was known to be located (Lowrie et al., 1987). The uninjured control left side of the block was marked for identification by penetrating the dorsal horn with a micropin along the length of the block.

Each individual block of tissue was then mounted on a freezing microtome (Pelcool). Serial sections of 50 um thickness were cut and immediately reacted for HRP histochemistry using a modified Hanker-Yates method (Hanker et al., 1977. For a detailed protocol see Appendix III).
The sections were then mounted on gelatinised slides and dried for 24 hours at 37 °C, after which they were counterstained with gallicyanin (Culling, 1963) between 15 to 25 minutes until the nucleolus was well stained and the Nissl substance lightly stained. Coverslips were placed on the slides using Permount and the slides allowed to dry for 24 hours.

5.2.6. Microscopy.

The stained sections were examined under a light microscope for the HRP reaction product in the motoneurones on both the operated (right) and control (left) sides of the spinal cord. The precise location of the motoneurone pool was confirmed (Nicolopoulos-Stournaras and Iles, 1983; Lowrie et al., 1987). The labelled cells were localised in the central area of the grey matter within the ventral horn and only those were counted whose nucleolus was visible. Labelled cells found outside the soleus pool as defined above were discounted and considered to be due to spread of HRP.

Motoneurones that were labelled could be distinguished from unlabelled cells. The nucleolus in each cell was counter-stained with gallicyanin to ensure that each labelled cell was counted only once.

Only those cells that were labelled and in which the nucleolus could be clearly seen were counted. In those labelled cells where the cytoplasm was very densely "packed" with granules and the nucleolus could not be distinguished, sections immediately rostral and caudal were reviewed in order to make sure that each cell was counted only once.
5.3 Results.

5.3.1. Localisation of labelled motoneurones.

The motoneurones innervating the soleus muscle were labelled retrogradely with the HRP and were located in lumbar segments L4 and L5. Rostro-caudally the majority of labelled cells were found in the L5 segment. This localisation was in agreement with the results of previous studies (Nicolopoulos-Stournaras and Ilies, 1983; Burls, 1987; Lowrie et al., 1987). Figure 5.2 A illustrates the localisation of the pool of labelled motoneurones to the soleus muscle in a normal animal. Even at low magnification, individual motoneurones can be distinctly identified. B and C of Figure 5.2 show labelled motoneurones at higher magnification. The HRP reaction product appears as dark distinct granules within the cytoplasm.

5.3.2. Motoneurone numbers in the normal soleus pool.

The mean number of motoneurones counted after injection of HRP into normal soleus muscles or the contralateral control soleus muscles of those animals which had had a sciatic nerve crush at birth was 56.8 ± 1.8 (S.E.M.). This mean compared well with previous observations using HRP to label motoneurones to the soleus muscles (Burls, 1987; Lowrie et al., 1987).

The motoneurone counts in this study were also in agreement with those found in other studies which used different methods to estimate the number of motoneurones supplying the soleus muscle. For example Zelená and Hník (1963) reported 54 motoneurones on the basis of axon counts in de-afferented nerves. Andrew et al., (1975) counted 52 motoneurones using conduction velocity of efferent fibres. They found 22 gamma and 30 alpha fibres supplying the soleus muscle in the rat.
Figure 5.2.
This figure shows an example of HRP filled motoneurones supplying the Soleus muscle in the lumbar spinal cord of an adult rat. (A) shows the localisation of the pool. (B) and (C) show HRP filled motoneurones at higher magnifications. In (C), the HRP granules can be clearly seen in the cytoplasm. In addition, the nucleus and nucleolus of the filled motoneurones can be clearly identified.
5.3.3. Effects of reducing the Calcium concentration around the site of injury in animals which had had sciatic nerve crush at birth.

In 3 animals, the right sciatic nerve was crushed at birth. The soleus muscles of these animals were injected with HRP two months later and their motoneurone pools visualised as already described. It was found that the mean motoneurone count on the operated side was 17 ± 0.33 (S.E.M.) and that of the contralateral control pool was 55 ± 6 (S.E.M.). Results from a previous study (Lowrie et al., 1987) in which the same operation had been carried out showed that mean motoneurone number on the operated side was 17 and that on the contralateral control was 55 (not significantly different from normal or control values found in this study using a Students' t-test).

The mean motoneurone number after treatment with BAPTA on the operated side was 24.5 ± 1.93 (S.E.M., N=10). The mean number of motoneurones counted on the contralateral control side was 58 ± 1.9 (S.E.M. N=10). It would appear that after treatment with BAPTA and sciatic nerve crush at birth more motoneurones were present than survived after sciatic crush at birth alone (17 in both this study and that of Lowrie et al., 1987). This is shown in Figure 5.3.

5.3.4. Effects of NaCl around the site of injury on motoneurone numbers in animals which had had nerve crush at birth.

In order to ensure that it was treatment with BAPTA around the tip that caused the slight increase in motoneurone number, and not the presence of the silicone rubber strip, NaCl containing silicone strips were placed alongside the regenerating nerve in 3 animals. The results obtained from these animals 2 months later when the motoneurones to the soleus muscles were visualised with HRP were not different to
Figure 5.3.

The mean number of motoneurones supplying the left or right soleus muscles in animals from 3 experimental groups are summarised in this Figure. The bars on the left of each pair show the number of motoneurones supplying the left soleus muscles whose nerves were uninjured. These served as contralateral controls within each animal. The hatched bars on the right in each pair show the mean number of motoneurones supplying the right soleus muscles which had had their sciatic nerves crushed at birth. These were referred to as the operated side in each animal. In 10 animals, in addition to the sciatic nerve being crushed at birth, a BAPTA containing silicone rubber strip was placed alongside the site of the crush 3 to 4 days later. Mean motoneurone number after such treatment is shown in the hatched bars on the right in Group A.

Group B and Group C show mean motoneurone number from animals which had sustained right sciatic nerve crush at birth. The hatched bars on the right in each of these pairs show the mean number of motoneurones that survived the injury. Group B shows results obtained in this study and Group C shows those obtained in a previous study. It is clear from the Figure that the mean number of motoneurones surviving sciatic crush was slightly increased by treatment with BAPTA. Comparing the results of 2 separate studies, it is also clear that there was no significant difference in mean motoneurone number seen after sciatic nerve crush at birth alone (Groups B (N=3) & C (N=4)).
mean motoneurone number

Group A  Group B  Group C

control  operated

treatment
those found in the present and previous studies where the regenerating nerve was left untreated. These are summarised in Figure 5.4. Mean motoneurone number on the right side (operated) after sciatic nerve crush at birth and treatment with NaCl was 17 ± 0.33 (S.E.M.) and that on the contralateral left (control) side was 56 ± 7 (S.E.M.).

Since there was no difference between the number of motoneurones supplying the soleus muscles in animals with no treatment and those with NaCl treatment after sciatic nerve crush at birth, the results of these two groups were pooled together and served as a control group. In each animal motoneurone numbers on the operated side were expressed as percentages of those of their contralateral controls for all the groups. Mean percentages of motoneurones on the operated sides of the control group and the BAPTA treated group were compared. These are summarised in Figure 5.5. The filled bar is the mean percentage after treatment with BAPTA. The hatched bar is the mean percentage of the pooled group of no treatment or sodium chloride treatment after sciatic crush at birth. Although this Figure indicates that after treatment with BAPTA more motoneurones survived, this increase was not statistically significant.

5.3.5 Effects of treatment with BAPTA around the regenerating nerve:
A comparison between motoneurone number and recovery of muscle function.

Lowrie (1987), has found that treatment of the regenerating nerve with BAPTA resulted in significant functional recovery of the reinnervated soleus muscles. After nerve crush at birth, when the injured nerves were treated with sodium chloride, reinnervated soleus muscles recovered 20% maximal tetanic tension of that produced by
**Figure 5.4.**
The mean number of motoneurones supplying the Soleus muscle on the control (empty bars) and operated (hatched bars) is shown. In Group A, in addition to the sciatic crush a NaCl containing silicone rubber strip was placed alongside the injured nerve. In group B, the sciatic nerve was crushed at birth but no subsequent treatment was given.
mean mn. number

Group A Group B

treatment

control operated
**Figure 5.5.**

This Figure compares the mean motoneurone numbers expressed as a percentage of contralateral control (which was the left side of each animal). The filled column shows values obtained from animals that had their crushed sciatic nerves treated with BAPTA. The hatched column shows pooled values obtained from animals with no treatment or NaCl treatment after sciatic nerve crush at birth.
operated % control

Group A  Group B

treatment
their contralateral control muscles (Lowrie et al., 1987; Lowrie 1987). This was in agreement with the results of the present study in which although the increase in motoneurone number on the operated side after treatment with BAPTA was not statistically significant, it nevertheless followed a similar pattern to the increased recovery of tension seen in the soleus muscles. The results of the 2 studies are summarised in Figures 5.6 and 5.7. Mean motoneurone number expressed as a percentage of contralateral control after sciatic nerve crush only, or treatment with NaCl or BAPTA are shown in Figure 5.6. Mean maximal tetanic tension expressed as a percentage of contralateral controls taken from Lowrie, (1987) are shown in Figure 5.7.

The improvement of muscle function in the BAPTA treated animals was greater than that of motoneurone survival. It is therefore unlikely that the effect of BAPTA on muscle recovery can be accounted for entirely by better motoneurone survival.
Figures 5.6. & 5.7
The number of surviving motoneurones supplying the Soleus muscle after sciatic nerve crush at birth and treatment with or without BAPTA or NaCl is expressed as a percentage of the contralateral control side and is shown in Figure 5.6. The filled bar is the mean from animals treated with BAPTA (Group A) and the two hatched bars are of means from animals with either NaCl (Group B) treatment or without treatment (Group C).

The values shown in Figure 5.7 are from a parallel study on the recovery of the Soleus muscle after sciatic nerve crush at birth with or without treatment with either BAPTA or NaCl (Lowrie, 1987). The maximum tetanic tension produced by reinnervated Soleus muscles is expressed as a percentage of their contralateral control muscles. The filled bar represents the mean maximum tetanic tension produced by reinnervated Soleus muscles treated with BAPTA (Group A). The two hatched bars are mean maximum tetanic tensions produced by reinnervated Soleus muscles after NaCl treatment (Group B) or without any treatment (Group C). After sciatic nerve crush at birth the recovery of tension in the reinnervated Soleus muscle was significantly greater as a result of treatment with BAPTA (p<0.001), than after NaCl treatment or no treatment.
5.4 Discussion.

5.4.1. Reliability of retrograde labelling with HRP.

A. Uptake and Transport.

The number of motoneurones labelled with HRP in this study compares well with the findings of previous studies (Nicolopoulos-Stournaras and Iles, 1983; Burl, 1987; Lowrie et al., 1987). However, this method of labelling cells is subject to many variables. Factors that affect the uptake and retrograde transport of the enzyme, the fixative, the degree of enzymatic activity of the HRP at the time of its reaction for demonstration and indeed the method of visualisation all may affect the results (for review see Mesulam, 1982). These variables would be expected to affect both the control and operated sides of the spinal cord equally and would not affect the relative numbers of labelled motoneurones on either the control or operated sides. The modified Hanker-Yates (Hanker et al., 1977) method was preferable to that of Mesulam (1982) for counts of the somas of motoneurones for two reasons: a) It has a granular deposit of the HRP molecules within the cytoplasm which makes the counting of cell-bodies much easier.

b) The nucleoli stain up very darkly in otherwise clear nuclei. This minimised errors of double counting one cell. Nevertheless the variability of uptake of HRP does imply "noise" within the system.

B. Spread of HRP.

In several experiments there was evidence of spread of HRP. This is known to occur when HRP is injected intramuscularly (Janjua and Leong, 1981; McHanwell and Biscoe, 1981). However, since the pool of soleus motoneurones is relatively well defined, cells outside it are easily identifiable and can be excluded.
Some experiments were done to reduce spread using lower concentrations of HRP such as 10% or 12% which had been used in previous studies to label the motoneurones of the soleus muscle (Lowrie et al., 1982;1987). However, with concentrations of 10% or 12%, there was a danger of underestimating the labelled pool since some motoneurones within it remained unlabelled or were only faintly labelled. It was thus thought best to use a 15% concentration in this study.

5.4.2. Possible explanations for motoneurone loss in young neuromuscular systems.

Previous investigations studying motoneurone loss either during normal embryonic development or after neonatal nerve lesions suggested two possible explanations for the loss.

Embryologists have explained the motoneurone loss as a result of the absence or insufficiency of a trophic factor released from the target (see Oppenheim, 1989 for review).

Morphologists working on mammalian nervous systems have correlated motoneurone loss following neonatal peripheral nerve injury to the intrinsic shortcoming of immature motoneurones. Metabolic characteristics of young motoneurones such as their inability to respond to injury with reversible chromatolysis have been blamed for their degeneration and death (Romanes, 1946; Hess, 1957; La Velle, 1964; Torvik, 1972; see also Lieberman, 1974).

Despite the extensive literature on the effects of nerve injuries on motoneurones (see Chapter 1, General Introduction) the role of the environment around the growth cone and its effect on the soma has not been considered. By attempting to modify this environment around the regenerating growth cone, the present study has indeed indicated a
modest improvement in motoneurone survival after neonatal nerve injury. Consistent with results of previous studies, those of the present study demonstrate the importance of interaction with the target for motoneurone survival (Lowrie et al., 1982;1987; Kashihara et al., 1987).

Although the results of the present study indicated that chelating calcium around the regenerating growth did increase the number of motoneurones that survived this improvement was not statistically significant for only 10% more motoneurones survived after BAPTA treatment than after NaCl or no treatment as a result of sciatic nerve crush at birth.

This finding therefore cannot explain the results of Lowrie (1987). Using the same model, (of sciatic nerve crush at birth with BAPTA or EGTA alongside the nerve 3 to 4 days later), they found better recovery of tension of the soleus muscle (50% of contralateral controls) than without treatment (20 to 30% of contralateral control cf. Figures 5.6 and 5.7).

This was one of the reasons for studying motoneurone survival after such treatment. However, it is possible that those motoneurones that survived could have had axons that regenerated quicker because of BAPTA hence this would certainly have been beneficial to muscle function. In chapter 2 of this thesis, on the early stages of reinnervation, it was shown that the soleus muscle was severely affected by denervation but recovered rapidly upon reinnervation (see also Lowrie et al., 1982;1984;1987).

The motoneurones supplying the soleus muscle that survived after the neonatal nerve injury and treatment with BAPTA (42%) may have
taken on larger peripheral fields than they normally would have and thus reinnervated muscle fibres whose motoneurones may not have survived the injury.

How then has BAPTA worked to improve muscle recovery without appreciably affecting the number of surviving motoneurones after nerve injury at birth?

In the Introduction (see section 5.1) to this chapter an analogy was made between the growth cones of regenerating nerves and those of neurites in culture. It has been found that calcium does indeed inhibit the elongation and motility of growth cones of neurites (see Kater et al., 1988 for a review).

Another explanation which may work in concert with those offered in the Introduction to this chapter comes from experiments on growth cones of regenerating axons and their cytoskeletal structure. The successful regeneration of axons following nerve injury is dependent among other things, on the maintenance and re-establishment of growth of the axonal cytoskeletal substructure within the intact portions of axons. Therefore, there is a great need for reordering the priorities in protein synthesis and axonal transport for regenerating neurons.

How then does calcium play a role here? Stability and self-assembly of mammalian neurofilaments has been demonstrated in vitro (see Schlaepfer, 1983). Their susceptibility to calcium was first seen in rat peroneal nerves minced into segments and exposed to calcium before examining them under the electron microscope (see Schlaepfer, 1983). The presence of calcium activated neutral protease (CANP) in the axoplasm would account for the disintegration of neurofilaments (Schlaepfer, 1979; 1983) in pathological conditions. Activation of this protease in nerve terminals could account for the breakdown and
turnover of neurofilaments in uninjured nerves. Recycling of neurofilament degradation products could represent an important feedback mechanism regulating their reproduction. The CANP is known to act at nerve terminals in immature animals in which more than one neurone innervates one muscle fibre (see O’Brien, 1983 for review). It has been postulated that the calcium which activates CANP is dependent on potassium to open voltage-gated channels to enter the nerve terminal (Connold et al., 1986; Evers, 1987; Vrbova et al., 1988; Connold, 1989).

After axonal damage the calcium almost certainly enters the proximal stump and could activate this neutral protease which destroys the neurofilaments and by reducing the calcium as in the present study, their assembly may be enhanced. Thus, chances of a moderate survival rate of the soma and the possibility of enhancement of the rate of growth of the regenerating axon both could explain the relatively good recovery of muscle function (Lowrie, 1987) and the small increase in motoneurone number seen after BAPTA treatment following nerve crush at birth (this study).

Llinas (1979) suggested that the presence of prominent calcium conductances in developing neurones may be associated with the role of calcium as an intracellular 2nd messenger controlling growth and maturity (see also Kater et al., 1988).

A nerve injury at birth may disturb the delicate balance that exists between ions and may cause disruption not just at the point at which injury is inflicted, or at the nerve terminal, but also in the soma of the injured neurone. Perhaps reduction of calcium at the regenerating growth cone may be beneficial to motoneurone survival.
after neonatal nerve injury provided all other variables are controlled. The present results could be best explained by suggesting an increased rate of regeneration to account for muscle improvement.

5.4.3 Possible future experiments.

1. It is possible that only a few motoneurones were saved by the treatment with BAPTA because the amount of calcium reduction was not enough. This could be easily tested by prolonging the calcium reduction around the growth cones by introducing a second silicone rubber strip containing BAPTA at the site of crush 7 days after the injury.

2. The rate of regeneration of sciatic nerves treated with BAPTA could be tested to see if indeed the reduction in calcium concentration around the regenerating growth cones caused them to reach their target quicker than if they had not been treated.

3. The first stages of functional recovery of reinnervated muscles could be tested using the criteria used in Chapters 2 and 3 in this thesis in order to see when exactly the first reinnervating axons reach their targets.

In conclusion then, briefly reducing the calcium concentration around the growth cones of young regenerating sciatic nerves seems to increase slightly motoneurone survival. The mechanisms as to how this occurs are still not clear. Nor is it clear why young motoneurones are so dependent on interaction with their targets or why they die if disconnected from their targets. This chapter has only attempted to study this process and the results suggest that the most likely mechanism of improved muscle recovery is an increased rate of regeneration which minimises the period of denervation between young neurones and their targets at this vulnerable stage. Consequently, the
chances of mismatch between the rate of growth of the motoneurone and its target is also minimised.
CHAPTER 6.

GENERAL DISCUSSION.

Functional interaction between neurones and their targets is known to be an important factor in their development and maturation. Stabilization of synaptic connections also appears to require this functional interaction and appears to be important for the development of adult behavior patterns (Young, 1951; Wiesel and Hubel, 1963; Stent, 1973; Changeux and Danchin, 1976; Wiesel, 1982; ). The neuromuscular system has provided neurobiologists with an accessible model for the study of functional interaction during development. Because of easy access to skeletal muscles and their innervation, much information has been collated as to the various aspects of their function and development (see Chapter, 1, General Introduction). In addition, it has also been relatively easy to alter both the inputs and outputs to the system. From the classical cross-innervation studies of Buller et al., (1960), it appears that adult muscle can certainly adapt to different patterns of innervation and evidence from studies on temporary denervation has shown that both motoneurones and muscles return to normal once they are reconnected (Foerhing et al., 1986; Lowrie et al., 1987; see also Chapter 1, General Introduction).

However, several studies on the neonatal neuromuscular system have shown that although adaptive changes occur upon reinnervation after temporary disruption between motoneurones and muscles, these changes tend to be long lasting and cannot be easily altered. Additionally, the recovery of both motoneurones and muscle remains impaired (Romanes, 1946; Zelena and Hnik, 1963; Lowrie et al., 1982; 1984; Navarrete & Vrbova, 1984; Kashihara et al., 1987; Lowrie et al., 1987).

Some of the experiments described in this thesis were an attempt
to minimise the effects of temporary interruption of neuromuscular interaction in young mammals (see Chapters 3 and 5). In addition, the experiments presented here have also investigated the long lasting effects of early postnatal injury, in order to have a better understanding of different reactions of fast and slow muscles and their motoneurones to temporary separation from their nerves (see Chapters 2 and 4).


At birth, slow muscles such as the Soleus contract and relax slowly and, after an initial increase in their speed of contraction, they become slowly contracting. Fast muscles such as the EDL muscle on the other hand show a continuous increase in their speed of contraction, as adult locomotor patterns begin to be established (see Close, 1964; Navarrete and Vrbova, 1983). Furthermore, the changing activity pattern of these muscles seen in development is reflected in the altered contractile properties of these developing muscles (Vrbova et al., 1985). This may be explained by the different functions required from presumptive fast and slow muscles. Fast, phasic flexor muscles such as the EDL are often required to perform sudden movements of varying force. Slow postural muscles such as the Soleus are required to maintain more or less steady levels of force.

In the rat, (as in many other species, see Chapter 1, General Introduction) if the contact between motoneurones and muscles is disrupted by sciatic nerve crush at birth, there is a loss of 70% of the motoneurones that supply the Soleus and EDL muscles (Romanes, 1946; Zelená and Hník, 1963; Lowrie et al., 1987). This results in
atrophy of the muscles, a loss in their force production and an alteration in their contractile properties (Zelená and Hník, 1963; Lowrie et al., 1987). However, motoneurones in the rat sciatic pool become resistant to death if the nerve crush is performed at 5 days of age, but the muscles they innervate (particularly fast muscles such as EDL and TA) remain vulnerable to disconnection from them (Lowrie et al., 1982; 1987; Lowrie and Vrbová, 1984).

In Chapter 2 of this Thesis, some of the possible reasons for the selective impairment of fast EDL and TA muscles after nerve crush at 5 days were explored. Slow Soleus muscles upon reinnervation, had been shown to recover to 80% of the strength produced by normal age-matched controls after a similar injury (Lowrie et al., 1982; 1987; Lowrie and Vrbová, 1984). Therefore, it was thought that there may be a difference in the time of arrival of regenerating axons to fast EDL and TA and slow Soleus muscles. As can be seen from Figures 2.1 (b) and 2.2 (b), reinnervation of both EDL and Soleus muscles by the regenerating axons appeared to be simultaneous as judged from the morphological data. In addition, mechanical responses from both fast and slow muscles could be elicited at the same time, that is, 7 days after the initial injury.

Upon the onset of reinnervation, the slow Soleus muscle showed a steady increase in strength which correlated well with its increase in weight. However, the reinnervated fast muscles, TA and EDL did not increase in strength during this period of development. In fact, their recovery seemed to have reached a plateau by 15 days after the initial nerve injury that suggested only a gradual subsequent increase. Although there was no correlative decrease seen in the weights of reinnervated fast muscles (compare Figures 2.5 and 2.6) the rate at
which the weights did increase was reduced. This was in agreement with a previous study (see Figure 4, Lowrie and Vrbová, 1984) which showed that fast muscles began to lose fibres upon reinnervation after sciatic nerve crush at 5 days.

Why then is there a difference in the recovery of fast and slow muscles despite simultaneous reinnervation? The difference may have been due to the muscle’s ability to respond better to the neural activity from Soleus motoneurones than to that imposed by the motoneurones supplying the fast EDL and TA muscles. This was hypothesized by Lowrie et al., in 1982 and some evidence to support it was provided in several subsequent studies (Lowrie and Vrbová, 1984; Lowrie et al., 1988), and some of the results presented in this thesis. Despite the closeness of the peroneal nerve crush to the fast muscles (3mm away), their recovery of strength and weight was still not as good as that of the reinnervated Soleus after a sciatic nerve crush inflicted at the same age several mm further away (Lowrie et al., 1982;1987; Lowrie and Vrbová, 1984). In addition, the results presented in Chapter 3 have also shown that the TA muscle recovers less well than the EDL muscle. This is substantiated by the results of Lowrie and Vrbová (1984), who have found that loss of muscle fibres in fast muscles occurs upon reinnervation. Furthermore, they have found that early during the recovery period, TA in particular shows large areas of very small fibres stained weakly for NADH reductase. This indicates that the superficial part of the muscle remains uninnervated (Engel and Karpati, 1968; Shafiq et al, 1972; Tomanek and Lund, 1973).

Following reinnervation after neonatal sciatic nerve crush, fast
muscles appear to lose all of their large, pale, glycolytic fibres, since the mosaic pattern seen in normal muscles is lost (Lowrie et al., 1982; 1987; Lowrie and Vrbová, 1984). These large, pale muscle fibres make up 30% to 40% of the total fibres in normal fast muscle. Instead, the muscles are darkly stained and become very fatigue resistant and highly oxidative. In addition, it appears that the normal complement of slow muscle fibres in the fast muscles is restored upon reinnervation (Lowrie et al., 1988). These authors suggest that since no motoneurone loss occurs after an injury at 5 days (Lowrie et al., 1982), and there is a decrease in motor unit size (Albani et al., 1988), the loss of muscle fibres in each unit would be at the expense of fast muscle fibres (Lowrie et al., 1988). This result is in agreement with evidence from adult mixed muscles in which a larger proportion of slow muscle fibres was found upon reinnervation (Lewis et al., 1982; Foerhing et al., 1986; Albani et al., 1988).

The evidence cited above may explain the results obtained from reinnervated EDL and TA muscles after unilateral or bilateral peroneal nerve crush 9mm away presented in Chapter 3. However, they cannot explain the preservation of half of the normal complement of pale fibres in EDL muscles reinnervated after a peroneal nerve crush 3mm away from them. Nor can they explain the correspondingly near-normal Fatigue Index of these muscles. It would be interesting to investigate whether these pale fibres are preserved because of the brief period of separation of the muscle from its nerve after an injury close to it.

The change in muscle profiles is probably induced by an altered activity pattern of motoneurones. This is indeed the case. Reinnervated EDL muscles have been shown to have 2 to 3 times the aggregate EMG activity compared to normal age-matched control muscles.
This hyperactivity is also expressed as an enhanced reflex responsiveness to stimulation of ipsilateral and contralateral branches of the sciatic nerve as the results of Chapter 4 indicate. The reasons for this hyperactivity are at present difficult to understand and are open to speculation.

### 6.2. Effects of temporary separation from the target early in postnatal life on the motoneurone: Development of possible aberrant connections?

In young rats, the intrinsic electrophysiological properties and synaptic connectivity of motoneurones are still immature and incomplete. Synaptogenesis and descending and intrinsic spinal connections are complete only by the third week of postnatal life (Stelzner, 1982). Interneuronal synapses onto motoneurones are formed during early postnatal development (Vaughn and Grieshaber, 1973; Saito, 1979) and primary afferent monosynaptic connections are established just prior to birth (Kudo and Yamada, 1987). However, evidence from kittens indicates that with the growth of dendritic trees as a result of increased synaptic inputs and postnatal development, 50% of synapses onto motoneurones are eliminated by the first three weeks of life (Conradi and Ronnevi, 1975).

The electrophysiological properties of young motoneurones also are still immature. Thus, neonatal motoneurones have a higher input resistance and low maximum firing rate compared to adult motoneurones (Fulton and Walton, 1986) which is consistent with the low motor unit firing rate seen in young animals (Navarrete and Vrbova, 1983). Additional developmental changes in motoneurone properties may reflect the growth of the somato-dendritic growth of the cells as well as
changes in the type, density or distribution of ion channels (Spitzer, 1979; see also Poo, 1985; O’Brien and Fischbach, 1986). Thus, a prominent high threshold calcium conductance exists in addition to the fast inward sodium current which is the basis of the action potential afterdepolarization (Harada and Takahashi, 1983; Walton and Fulton, 1986). This high threshold calcium conductance is coincident with the period of increased and active growth of the motoneurone dendritic growth cones and peripheral field (Brown et al., 1976; Cummings and Stelzner, 1984). These calcium conductances are found to be specifically prominent in developing motoneurones and may be related to the growth processes triggered by calcium in its role as an important intracellular second messenger (Llinas, 1979; Kater et al., 1988). The results discussed in Chapter 5 of this thesis showing a small but insignificant increase of motoneurone survival as a result of excess calcium chelation around the growth cone may be related to this phenomenon of prominent calcium conductances.

Preliminary results on the electrophysiological properties of motoneurones innervating slow Soleus or fast EDL or TA show that within the first week after birth, both these motoneurone populations have marked afterdepolarization and afterhyperpolarization reflecting the presence of prominent calcium and calcium-activated potassium conductances (Navarrete et al., 1988). Partial spikes have also been seen to occur in immature motoneurones (Kellerth et al., 1971; Walton and Fulton, 1986). These properties reappear in adult motoneurones when they are disconnected from their targets (Kuno et al., 1974; Huizar et al., 1975). Axotomised adult motoneurones become more excitable than normal and
and the stimulation of single afferent fibres can result in "partial spikes" (Kuno and Llinas, 1970) which are apparently due to a high density of sodium channels (Sernagor et al., 1986). All these changes are reversed upon reinnervation of the target (Poerhing et al., 1986).

So what happens when the motoneurone is disconnected from its target at this early stage of development?

In rats, disconnection of motoneurones from their targets either by axotomy or nerve crush within the first 3 days of postnatal life, results in death of motoneurones (Romanes, 1946; Bueker and Meyers, 1951; Lowrie et al., 1987). It is separation from the target and the prevention of reinnervation that seems to cause death and not nerve injury per se (Kashihara et al., 1987). The extent of motoneurone death depends on the age of the animal and the type of injury, (Romanes, 1946; Lieberman, 1974; Schmalbruch, 1984; Lowrie et al., 1987;) so that, by five days of age no motoneurones die as a result of sciatic nerve crush (Lowrie et al., 1982).

Here it is interesting to note that excitatory amino acids have been shown to play an increasingly important role in neuronal cell death in several parts of the CNS (Coyle et al., 1981; Olney, 1983; Garthwaite and Garthwaite, 1986). They have also been implicated in several neurodegenerative disorders such as epilepsy (Meldrum, 1983) and ischaemia (Simon et al., 1984; Gill et al., 1987) all causing cell death. Glutamate receptors have also been found on embryonic chick motoneurones (O'Brien and Fischbach, 1986b). It is possible that young motoneurones may die as a result of neonatal nerve injury because of excess excitatory amino acid toxicity which may result because of their inability to cope with excess synapses on their soma. The reduction of synapses on the motoneurone cell body seen
during development (Conradi and Ronnevi, 1975) may fail to occur in the absence of the motoneurone's interaction with its target.

It may be hypothesized that the slight increase in the number of injured motoneurones after sciatic nerve crush at birth and treatment with BAPTA may be due to enhancing regeneration and re-establishment of connections with the target, thereby allowing the reduction of excess synapses on the soma to proceed.

In new born rats, sensory neurones also die as a result of sciatic crush (Romanes, 1946; Zelená and Hník, 1963; Bondok and Sansone, 1984; Yip et al., 1984). This may result in the permanent loss of synaptic input to spinal neurones onto which they normally project. It may be that the vacated synapses are occupied by other inputs.

Several previous studies have explored the possibility that undamaged primary afferent neurones may be able to expand their monosynaptic connections to motoneurones to occupy those vacated by the loss of sensory neurones. For example, Eccles, Eccles and Shealy (1962), studied the effects of injury to the nerves supplying the deep peroneal muscles and medial gastrocnemius in kittens on monosynaptic activation of their motoneurones by afferent inputs from their own and other afferent nerves. They found that several months after reinnervation, the size of the monosynaptically elicited EPSP was reduced as a result of stimulation of the regenerated nerve. They thought that this may be due to degeneration of primary afferent neurones. However, they also found that there was no change in the extent of monosynaptic activation by primary afferent neurones from the intact nerve. Later observations on the deafferentation of chick embryos by lesions of the neural crest did not show development of
aberrant monosynaptic connections (Eide et al., 1982). In addition a recent study has shown that stimulation of Ia afferents from medial gastrocnemius nerves crushed in one-day-old rats elicited monosynaptic EPSPs in only 40% of the motoneurones supplying that muscle. Even when the EPSP was elicited, it was smaller than that of normal age-matched medial gastrocnemius motoneurones (Miyata et al., 1986).

The only report of the maintenance of aberrant monosynaptic connections to motoneurones is that by Eccles et al., (1960) in which they showed that following cross-reinnervation of peroneal muscles by the gastrocnemius nerve, some peroneal motoneurones could be activated monosynaptically by lateral gastrocnemius afferents. Thus, the increased activity and enhanced reflex responses seen in fast EDL muscles as result of neonatal nerve injury cannot be explained by an increase in monosynaptic activation of the regenerated motoneurones (see Chapters 3 and 4 in this Thesis).

Since the majority of inputs to motoneurones is not monosynaptic, but mediated largely by interneurones (Baldissera et al., 1981), it is possible that the results presented in Chapter 4 of this Thesis show an alteration of such polysynaptic reflexes. It may be that some interneurones that normally project to motoneurones may sprout to occupy the synaptic space vacated by lost projections. Examples of such plasticity have been demonstrated in different parts of the adult nervous system (Cotman et al., 1981; Mendell et al., 1984; Purves and Lichtman, 1985). In this context, in a recent study on crossed reflexes in the rat, a short-latency crossed EPSP was described in hindlimb motoneurones. This reflex occurred in response to stimulation of contralateral afferents (Edgley and Wallace, 1989). The reflex was thought likely that to be mediated by lamina VIII interneurones which
have been shown to project onto contralateral motoneurones (see Harrison et al., 1986). The fine tuning of synaptic activity within the spinal cord achieved by these interneurones during development may be upset by the temporary cessation of influences from the target as result of nerve injury, thus causing an enhanced reflex response seen in the fast EDL muscles in Chapter 4.

In addition, Havton and Kellerth (1987) have shown that axotomised adult motoneurones grow supernumerary axons which have the appearance of a functional synapse at the ultrastructural level. It may be that as a result of neonatal nerve injury, young motoneurones also make such superfluous contacts and maintain them.

It may be hypothesized therefore, that if the mechanism by which restriction of reflexes is achieved during development involves the retraction of expanded connections formed in early life (Saito et al., 1979), then the enhancement of reflex activity seen in our results after neonatal nerve injury may be due to the remnants of the neonatal reflex pattern.

Finally, it may be possible that the intrinsic properties of neonatal motoneurones may be altered as a result of neonatal nerve injury and this may account for the increased activity of motoneurones expressed as enhanced reflex responsiveness as the results of Chapter 4 indicate. It may also explain the prolonged afterdischarge seen in reinnervated EDL muscles in response to stimulation of the regenerated peroneal nerve (see Figure 4.4). It may be that as a result of neonatal injury some motoneurones become and remain more excitable than normal. As has already been discussed above, several parameters related to the excitability of the
motoneurone such as size, passive and active membrane properties, continue to develop postnatally (Fulton and Walton, 1986; Navarrete, Walton and Llinas, 1988). Their full maturation may depend on continued synaptic contact with the target. Some evidence does indicate that in the rat, after sciatic nerve crush at 5 days, motoneurones supplying the fast EDL muscle become permanently smaller than those from normal weight and age-matched control animals (Lowrie et al., 1982). This reduction in size seems to agree with the increase in aggregate EMG activity of its muscles. (Navarrete and Vrbova, 1984). The results presented in Chapter 4 of this thesis confirm the above findings by showing an enhancement of the reflex activity of the muscles supplied by these neonatally injured motoneurones. It could be hypothesized that a permanent change in the intrinsic activity of the motoneurone or in its synaptic input occurs as a result of neonatal nerve injury as an explanation for the results presented in this thesis, particularly those presented in Chapter 4 which show the enhancement of normally weak reflexes (see also Navarrete et al., 1986).

In conclusion, the results presented in this thesis have confirmed those of previous studies suggesting that nerve-muscle interaction is a necessary prerequire at least in mammalian systems, for the development of motoneurone and muscle activity.
APPENDIX I.

CHOLINESTERASE & SILVER FOR YOUNG MAMMALIAN ENDPLATES.

1. Fix slightly stretched muscles in buffered 4% formaldehyde at 4 C for two hours.

0.2M Phosphate Buffer : solution A : 0.2M NaH₂PO₄ 23ml
: solution B : 0.2M Na₂HPO₄ 77ml
: adjust pH to 7.4 if necessary.

Fixative : Paraformaldehyde (EM grade) 4g
: distilled water 40ml
: heat to 60 C and add 4 drops of 4% NaOH to dissolve paraformaldehyde.
: cool.
: 1/2% CaCl₂ 1ml
: sucrose 5g
: DMSO 1ml
: 0.2M phosphate buffer 50ml
: distilled water to make 100ml.

2. Transfer tissue to washing solution for 1/2 hour at 4 C.

Washing solution : 0.2M phosphate buffer 50ml
: sucrose 5g
: 1/2% CaCl₂ 1ml
: DMSO 1ml
: distilled water to make 100ml.

3. Cut frozen sections at 25um into washing solution on ice. Sections or tissue may be held overnight in washing solution.
4. Incubate sections for one hour on ice.

    Incubation medium:  Acetylthiocholine iodide 10g
    : 0.1M Sodium hydrogen maleate 13ml
    : 100mM tri-sodium citrate 1ml
    : 30mM copper sulphate 2ml
    : glass distilled water 2ml
    : 5mM potassium ferricyanide 2ml
    : sucrose 3g

add solutions in order and mix well after each addition.

Maleate Buffer: Maleic acid (CH(COOH):CH.COOH) 1.16g
    : sodium hydroxide (NaOH) 0.22g
    : distilled water to make 200ml

adjust to pH 6.0 if necessary.

5. Transfer free floating sections to distilled water for 10 minutes
    (or hold overnight at 4°C).

6. Immerse in potassium ferricyanide for 5-10 minutes at room
    temperature. K$_2$Fe(CN)$_6$: 0.25g + distilled water 100ml.

7. Rinse in distilled water, two washes of 5 minutes each.

8. Transfer sections to absolute ethanol for one hour. Ensure that the
    sections lie flat; sections turn a grey-black-pink.

9. Transfer sections to distilled water, two washes of 5 minutes each
    (do not shake). The sections will spin flat on the surface of the
    first change of distilled water, so handle carefully one by one
    or else they will curl beyond repair.
10. Incubate sections in silver solution at 37°C for 20 minutes.

Silver solution: distilled water 100ml

: CaCO₃ 0.1g and double filter.
: CuSO₄•5H₂O 0.05g

Take 20 ml of the above solution and add to 2g of AgNO₃ and swirl to dissolve.

11. Rinse in large volume of distilled water for 5-60 seconds.

12. Immerse in reducer solution and control microscopically. Transfer to Petri dish of distilled water and follow the final development of the nerves and endplates. Wash for 10 minutes in large volume of distilled water.

Reducer solution: Na₂SO₃ 10g

: distilled water 100ml
: hydroquinone 1g.

13. Mount onto gelatinised glass slides and dry in oven. Dehydrate, clear and coverslip with DPX.

Notes: All solutions must be fresh.

: Silver precipitate contaminates sections if the silver solution is cloudy.
APPENDIX II.

SUCCINIC DEHYDROGENASE STAIN FOR OXIDATIVE CAPACITY OF MUSCLES.

Stock Solutions.

1. 0.1M Phosphate Buffer pH 7.6: a) Dissolve 1.42g \( \text{Na}_2\text{HPO}_4 \) in 100ml distilled water.
   b) Dissolve 0.468g \( \text{NaH}_2\text{PO}_4 \) in 30ml distilled water.
   Add (b) to (a) a little at a time until pH 7.6 is reached.

2. 1M Na succinate: 1.62g in 10ml buffer; keeps in 'fridge.

3. 15mM nitroblue tetrazolium: 246mg in 20ml distilled water. May need to warm up to 45°C to dissolve; keeps in 'fridge.

4. 0.1M KCN: 13mg in 2ml buffer. Make fresh.

5. 10mM phenazine methosulphate: 12.2mg in 4ml distilled water. Must be made up fresh. Very sensitive to light, so protect while making up working solution. If colour changes from yellow to green, discard.

Working Solution.

1. Take 32.8ml of 0.1M phosphate buffer pH 7.6
2. Add 2ml 1M Na succinate in buffer
3. Add 4ml 15mM nitroblue-tetrazolium
4. Add 0.4ml 0.1M KCN in buffer
5. Add 0.8ml 10mM phenazzine methosulphate
6. Filter and keep in dark bottle at 4°C.
Method.

1. Put few drops of working solution over sections on slide. Incubate at 37 C for 5 minutes.

2. Wash in 0.9% saline for 1 minute, then dehydrate in 70% acetone, 90% acetone, absolute alcohol and Histoclear for two minutes each. Mount in DPX. The background is clear and the SDH stained fibres are blue.
APPENDIX III.

MODIFIED HANKER-YATES METHOD FOR VISUALISING MOTONEURONES FILLED WITH HORSERADISH PEROXIDASE.

Solutions.

1. Millonig's phosphate buffer : \( \text{NaH}_2\text{PO}_4\cdot2\text{H}_2\text{O} \) 19.08g in 903.7ml

   : distilled water.

   : 1M NaOH 96.3ml (For 200ml 1M NaOH,

   : 8g in 200ml distilled water).

Adjust to pH 7.3 and make up to 1 litre with distilled water.

2. Cobalt/nickel solution : 1% cobalt chloride 300ml

   : 1% ammonium nickel sulphate 200ml

Make fresh and mix together just before use.

3. Cacodylate buffer : Solution A : 0.1M sodium cacodylate

   : (21.4g/500ml distilled water)

   : Solution B : 0.2N HCl (17.22ml 10N

   : HCl/litre distilled water

Add 500ml of solution a to 440ml of solution B. Adjust to pH 5.1-5.2.

Make up to 2 litres with distilled water (keeps in 'fridge).


   : (Sigma) 150mg

   : Na cacodylate buffer (pH 5.1-5.2)

   : 100ml

   : 1ml of 1% solution \( \text{H}_2\text{O}_2 \)

Make up just before use, discard after one hour.

5. Counterstaining solution, Gallocyanin : 0.3g Gallocyanin

   : Chromalum \((\text{CrK(SO}_4)_2\cdot12\text{H}_2\text{O})\)

   : 10g

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Dissolve chromalum in the water by heating. Add the Gallocyanin, bring to boil and allow to simmer for 20-30 minutes. Cool, add distilled water to the original volume and filter.

Method.
1. Perfuse animal through heart with 2.5% gluteraldehyde in Millonig's buffer, preceded by a short flush with saline or buffer. Remove spinal cord and post fix blocks for about 4 hours at 4°C, followed by 30% sucrose in Millonig's buffer at 4°C overnight (see also section 5.2 Methods in chapter 5).
2. Cut frozen sections at approximately 50um. Collect in Millonig's buffer.
4. Brief wash in distilled water. Rinse in 2 changes Millonig's of buffer, 10 minutes each.
5. React in Hanker-Yates solution 10 to 25 minutes, stopping before background darkens.
6. Rinse in 2 changes of Millonig's buffer, 5 minutes each.
7. Mount sections on gelatinized slides and dry overnight at 37°C.
8. Next day, brief wash in distilled water. Counterstain in Gallocyanin for 10 to 25 minutes, depending on age of the stain (the older the stain, the longer the counterstaining time).
9. Brief wash in distilled water. Dehydrate in 2 changes of 100% alcohol and 2 changes of Histoclear, 2 minutes each. Mount with Permount.
REFERENCES.


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